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TABLE OF CONTENTS

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iv

v

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STUDIES IN THE METABOLISM OF CRASSULACEAN PLANTS: CHANGES IN THE COMPOSITION OF *BRYOPHYLLUM CALYCINUM* DURING GROWTH

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Certain peculiarities in the chemical composition of succulent plants, especially those belonging to the family Crassulaceae, have long attracted the attention of plant physiologists and chemists. As early as 1815, HEYNE (4) noted that the leaves of *Bryophyllum calycinum* (then known as *Cotyledon calycina*) were intensely sour to the taste in the morning but lost their sour taste toward evening, becoming bitter instead. The outstanding chemical studies of this phenomenon were made 60 years and more ago by ADOLPH MAYER (7) who associated the changes in taste with reciprocal diurnal alterations in the concentrations, respectively, of titratable acid and of sugar. He also demonstrated that oxygen is liberated during the deacidification process in light, even when carbon dioxide is excluded, and recognized what he thought to be an isomer of malic acid.

An extensive literature on what is commonly referred to as the crassulacean type of metabolism has since appeared. The papers before 1933 have been carefully reviewed by BENNET-CLARK (1) [see also EVANS (3), and VICKERY and PUCHER (12)], and there have been a few more recent investigations, notably by WOLF (16, 17). The fundamental observations have been repeatedly confirmed, and many additional facts have been recorded; it cannot be claimed, however, that a great deal of light has been shed upon the chemical mechanisms involved, despite the manifest and widely recognized importance of the problem. Nevertheless, the development in recent years of reasonably satisfactory and accurate methods for the analysis of plant tissues and, more especially, the identification of MAYER's alleged isomer of malic acid (the so-called "crassulacean malic acid") as isocitric acid (5, 10, 11) have led to the hope that renewed study might reveal helpful information. In this series of papers, accordingly, the results of analytical investigations, chiefly of the leaves and stems of *Bryophyllum calycinum*,

are to be described; the present paper deals with the changes in composition that occur during normal growth. The experiments were carried out in order to see to what extent the changes in chemical composition of a plant which has a pronounced diurnal variation in organic acidity may differ from those of a plant, such as tobacco, which does not display this phenomenon.

Preparation of samples

Leaves picked from Bryophyllum plants derived originally from a single specimen were placed on moist sand in the greenhouse on September 1, 1940, and, 7 weeks later, the plantlets that had formed on the margins were separated and transplanted. On January 2, 1941, 30 young plants were again transplanted into sand in individual 1-gallon crocks and were thereafter flushed three times a week with a nutrient solution of the composition 0.001 M KH_2PO_4, 0.00225 M $Ca(NO_3)_2$, 0.0041 M $MgSO_4$, 0.0021 M KNO_3, 0.0043 M $Mg(NO_3)_2$ and which contained, in addition, 1 mg. per liter each of boron, manganese, and iron. The solution was adjusted to pH 6.0 by the addition of 0.4 ml. of 1.0 N sulphuric acid per liter. On March 27, 20 plants of uniform height and leaf size were selected and divided into four similar groups of five each. One of these groups was taken for the initial sample of the growth experiment (recorded as zero time).

The samples were harvested at 32, 62, and 95 days, on dates selected according to the weather, a bright cloudless day being chosen. The leaves were broken off at the junction with the petiole, the operation invariably being begun between 12:05 and 12:15 P.M. (standard time) in order to provide as great a degree of similarity as possible with respect to the stage in the deacidification process that had been reached in the different samples. The thick, mature, dark green basal leaves were kept separate from the thinner, smaller and lighter green upper leaves. The separation into groups was arbitrary and largely subjective but permitted comparisons to be made between the composition of mature and of relatively immature leaf tissue when the data are expressed in concentration units. Counts and fresh weights were taken and the tissue was then immediately dried in an oven at 80° C. in rapidly circulating air.

After removing the leaves, the stalk was cut at the sand level and the fresh weight, including the petioles, was determined. The tissue was then cut transversely into thin slices and likewise dried. The total time required until the tissues were placed in the oven was approximately 1 hour.

A similar procedure was followed on each of the sampling dates. However, it was necessary to discard 1 plant each from the 32- and the 62-day samples because it was less well developed than the others and had lost a few leaves; 2 plants of the 95-day sample were discarded for the same reasons. After being dried, all samples of tissue were weighed (crude dry weight) before being broken up, and were then powdered in a Wiley mill and subsequently preserved in closed containers. The fundamental data on the counts and weights are assembled in table I. All analytical determinations were

made upon these samples, the results being obtained as percentages of the crude dry weight and then computed in terms of grams, or milliequivalents in the case of organic acids, per single plant, or in grams per kilogram of original fresh tissue weight (*i.e.*, in concentration units), by means of factors obtained from the fundamental data. The one method of calculation provided a means of comparison between the composition of single plants at different stages of growth, the other permitted comparisons upon a concentration basis as growth progressed.

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TABLE I

FUNDAMENTAL DATA ON COUNTS AND WEIGHTS OF SAMPLES OF *Bryophyllum calycinum* PLANTS

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Growth period (days)	0	32	62	95
Sampling date (1941)	3/27	4/28	5/28	6/30
Number of plants	5	4	4	3
Number of mature leaves	33	68	124	149
Number of young leaves	69	139	187	140
Total number of leaves	102	207	311	289
Total number of petioles	57	65	85	60
Average length of stem (cm.)	17	39	67	90
Crude dry weight of mature leaves (gm.)	19.2	44.9	75.0	110.9
Crude dry weight of young leaves (gm.)	10.6	39.5	78.9	57.7
Crude dry weight of stems and petioles (gm.)	6.4	24.8	58.9	74.2
Crude dry weight of roots (gm.)	4.9	13.4	23.8	23.8
Fresh weight of mature leaves (gm./plant)	52.9	122.8	208.4	366.0
Fresh weight of young leaves (gm./plant)	25.4	110.2	203.2	181.9
Total fresh weight of leaves (gm./plant)	78.3	233.0	411.6	547.9
Fresh weight of stems and petioles (gm./plant)	14.1	77.1	181.6	267.7

Analytical methods

Many of the analytical methods employed in this laboratory have been specially developed and are described in journal papers. A summary of these methods, with full references, is to be found in a recent bulletin from this Station (15).

Growth in terms of organic solids and ash

The data for the fresh weight, the organic and inorganic solids, and the water content of both leaf and stem tissue are plotted in terms of grams per single plant in figures 1 to 3. During the period of observation, the growth rate of both the leaves and stems was essentially linear with respect to increase in fresh weight although that of the stems appears to have been slightly accelerated with respect to the rate of accumulation of organic solids as the plants increased in size.

A comparison of the rate of growth of the Bryophyllum plant with that of the tobacco plant is of interest. Data obtained during the season 1933 (13) for tobacco plants grown in the field under conditions of commercial production indicated that, 26 days from transplanting, the plants had become well established. At this point they were just passing into a period about 35 days long during which they grew at an essentially linear rate, and

the leaves were found to increase in fresh weight at an average rate of 15 grams per day, the stalks at about 16.6 grams per day. These figures are to be contrasted with an average rate of 5.7 grams per day for the leaves and 2.7 grams per day for the stems of the Bryophyllum plant over the 95-day
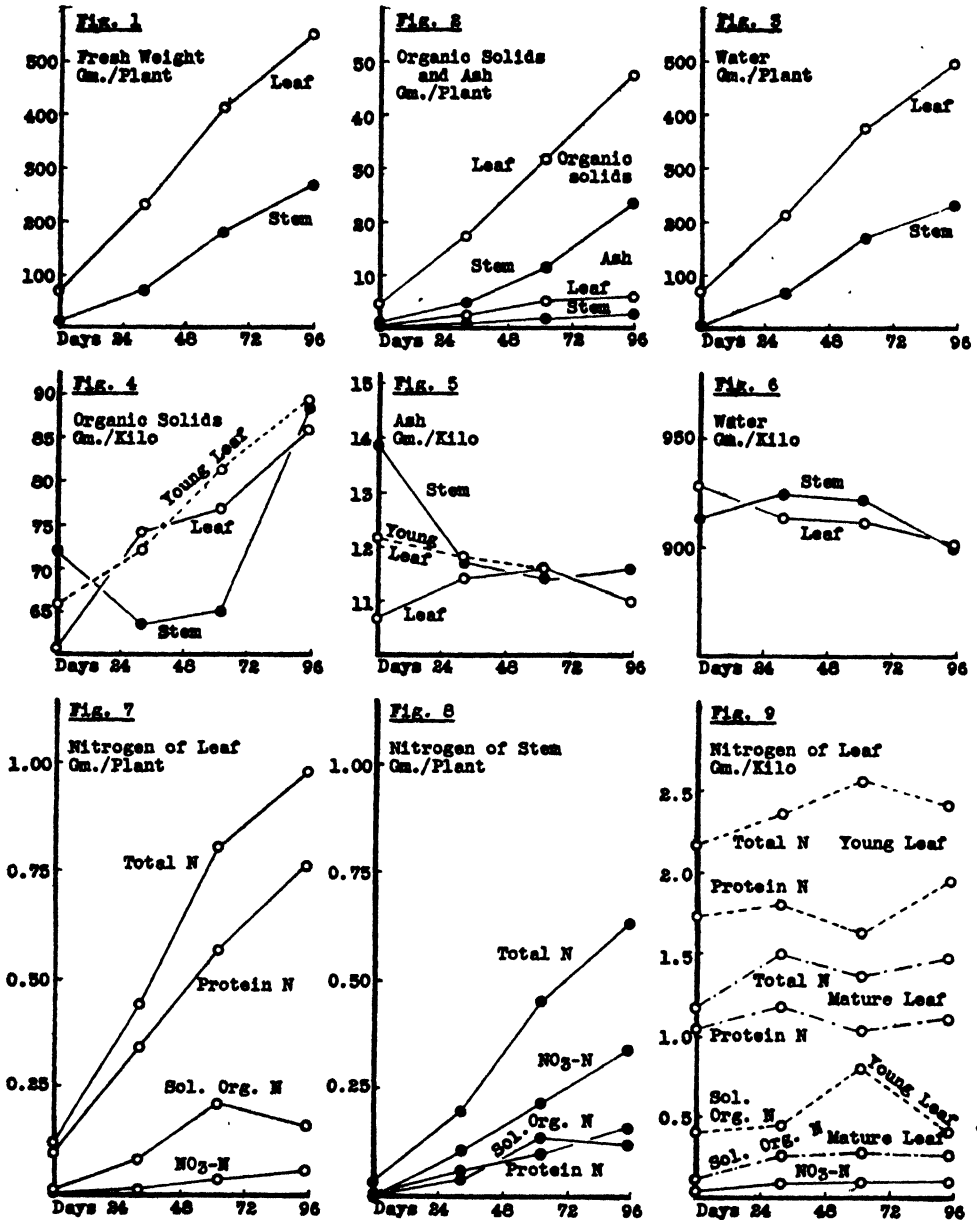
period of the present experiment. Clearly the tobacco plant grows much more rapidly than the Bryophyllum plant when compared at presumably analogous points in the course of their respective development.

Figures 4, 5, and 6 show the organic solids, the ash, and the water content of the leaf and stem tissue in concentration units. Although the data for the organic solids of the total leaf tissue indicate a somewhat irregular

increase in concentration, those for the young upper leaves (broken line) follow a nearly linear course. In the stem, on the other hand, the concentration of organic solids decreased at first and then increased rapidly.

The concentration of the ash constituents in the young leaves was higher than the average for the whole mass of leaf tissue in the plants at the start, but the difference became inappreciable as growth progressed. The concentration of ash constituents in the stem decreased rapidly at first, but then levelled off and remained essentially constant after 32 days of growth.

Figure 6 shows that the water content of both leaf and stem tissue remained between the limits 90% and 93% of the fresh weight throughout the experiment. The data for the leaf suggest a small continuous decrease in water content with age whereas the stem increased slightly at first and then decreased. Although this plant is classified as a "succulent," the water content of the leaves does not differ greatly from that of tobacco. Leaves from young growing tobacco plants contain from 89% to 90% of water.

Growth in terms of nitrogenous components

The rate of accumulation of total nitrogen, protein nitrogen, soluble organic nitrogen, and nitrate nitrogen is shown in figure 7. Growth, as measured by total nitrogen, gives a curve that is slightly concave to the time axis although, as measured by organic solids (fig 2), the curve is linear. It may be inferred that, toward the end of the period of observation, the rate of synthesis of organic substances free from nitrogen gradually increased relatively to the rate of synthesis of nitrogenous organic substances. Nevertheless, the rate of synthesis of protein in the leaf is shown by a curve that departs very little from linearity. The curve for the soluble organic nitrogen (i.e., soluble nitrogen exclusive of nitrate), however, indicates that the rate of accumulation of this group of substances was not maintained in the last interval between observations in spite of the fact that the storage of nitrate nitrogen in the tissue increased regularly. The plants were clearly living under conditions of liberal, if not luxury, supply of nitrate nitrogen, and the alteration in the rate of accumulation of soluble nitrogenous organic substances reflects an alteration in the general course of the metabolism as the plants matured. As is shown in figure 9, the fluctuations in the concentration of the soluble nitrogenous substances in the young upper leaves go far to account for this change; the concentration in the lower older leaves remained essentially constant.

The data in figure 8, showing the rate of accumulation of nitrogen in the stem, are plotted on the same scale as those of figure 7. Although the total nitrogen of the stem increased almost linearly, at each stage of observation slightly more than one-half of the nitrogen was present as nitrate, and only about one-quarter as protein. Both nitrate and protein nitrogen accumulated at a steady rate. However, the soluble organic nitrogenous substances decreased in the stem during the last period of observation as they did in the leaf, again reflecting the change in the general course of the nitrogen metabo-

lism as the plants approached maturity. The large quantity of nitrate suggests a storage function for the stem tissue. Analogous observations have been made for both tomato (2) and tobacco (13) plants, and the phenomenon is doubtless common.

The concentration of the total nitrogen and of the protein nitrogen in both the young and the mature leaves is plotted in figure 9, together with the concentration of the soluble organic nitrogen. There is a marked contrast between the young and the older leaves; the young leaves were much richer both in nitrogen and in protein and the curves for the latter component, although somewhat irregular, run parallel to each other for most of the period studied. The curve for the concentration of organic soluble nitrogen, like that of the total quantity per plant (fig. 7), reveals a moderate increase followed by a decrease in the last period of observation. The nitrate nitrogen in the whole leaf tissue increased in concentration.

The relative decrease in soluble organic nitrogenous substances coupled with an increase in the protein nitrogen in the plants 95 days old as compared with those 62 days old is of considerable interest. The change is evident in the data for absolute quantities per plant as well as in those expressed on a concentration basis. Examination of the detailed data for the young and for the more mature leaves shows that, during the last interval between observations, the concentration of protein nitrogen in the mature leaves increased very little while that in the young leaves increased materially. However, the concentration of soluble organic nitrogenous substances in the mature leaves remained constant while that in the young leaves decreased. Thus, in the mature leaves, there was a nearly constant relationship between the concentrations of soluble nitrogenous substances and the protein, although both increased in absolute amount per plant. In the young leaves, on the other hand, an increase in the concentration of the protein was associated with a marked decrease in the concentration of the soluble nitrogenous substances.

The careful review by PETRIE (9) of the literature of protein synthesis in the plant suggests that protein synthesis is correlated with the level of amino acids in the cell solution but that the relationship is not a simple one; other factors, especially the respiration rate and, accordingly, the rate of liberation of energy, play a part. The present data are not in conflict with Petrie's view of a "drifting steady state" with respect to the relationship between the protein and the amino acids of the plant cells. A reciprocal relationship between the concentration of the soluble nitrogen and of the protein nitrogen is evident in the young leaves although not in the more mature ones. This suggests that the high concentration of soluble nitrogen at 62 days may have been a factor in the relative increase in the concentration of protein nitrogen observed later in the young leaves of the plants.

Figure 10 shows the changes in the concentration of the nitrogenous substances in the stem and should be compared with figure 8 which shows the absolute quantities. There was a sharp increase in the concentration of the

total nitrogen at the start, and much of this arose from the increase in nitrate concentration. Subsequently, the concentration of the nitrate diminished slightly and then remained nearly constant. Nitrate nitrogen made up about one-half of the total nitrogen of the stem throughout the period studied. A high concentration of protein nitrogen is associated with a low concentration of soluble nitrogen in the youngest plants, but this situation is reversed in those 62 days old. In the 95-day-old plants, the concentration of protein nitrogen had increased slightly while that of the soluble nitrogen diminished materially. The reciprocal relationships in the stem are thus analogous to those observed in the young leaves.

Growth in terms of organic acids

The composition of the leaf tissues with respect to organic acids is shown in figure 11 and that of the stem in figure 12. Bryophyllum leaves are extraordinarily rich in organic acids; the present samples contained from 26% to 32% of the organic solids as organic acids, and these substances are, accordingly, major components from the quantitative point of view. However, the diurnal variation in the organic acids present makes it necessary to specify the time of collection before quantitative statements have precise meaning. During the night, the quantity of acid, especially of malic acid, increases but this process is reversed as soon as the leaves are illuminated so that the level of organic acids has greatly decreased by midday or early afternoon. The present samples were collected at a few minutes past noon on bright sunny days; had they been collected in the early morning, malic acid would doubtless have been the predominating acid component. The smoothness of the curves in figures 11 and 12 suggests that the precautions taken with respect to sampling time were effective.

The total acidity of the leaves, like the organic solids and the protein nitrogen, follows a nearly linear course throughout the experimental period, and this is also true of the malic acid. The rate of accumulation of isocitric acid declined as the plants matured. Citric acid was present in only moderate quantities and the increase during growth was small. Oxalic acid was present in the leaf tissue only in traces, the total amount even in the 95-day plants being so small as to be scarcely apparent on the scale of the diagram.

Organic acids make up a smaller relative proportion of the stem tissue than of the leaf. Figure 12 is plotted on a scale of $3\frac{1}{3}$ times larger than figure 11 in order to show the changes. It will be recalled that the organic solids of the stem amounted to about one-half those of the leaves (fig. 2) but that the stems of even the oldest plants contained less than one-fifth as much organic acid as the leaves. This is the reverse of the situation observed in the rhubarb plant where the petiole was found to be far richer in organic acids (14) than the blade.

The general picture of the rate of accumulation of organic acids in the stem is, however, not unlike that in the leaves. The total organic acids increased along a straight line curve for 62 days, but the curve then turned

upwards as the relative quantity of organic solids increased in the more mature stems. The curves for "isocitric" acid and malic acid conform. Neither citric nor oxalic acid was present in more than traces in the stem tissue.

Fig. 10. Nitrogen of Stem Gm./Kilo

Fig. 11. Organic Acids of Leaf M.e./Plant

Fig. 12. Organic Acids of Stem M.e./Plant

Fig. 13. Organic Acids of Leaf M.e./Kilo

Fig. 14. Organic Acids of Stem M.e./Kilo

Fig. 15. Organic Acids as % Organic Solids
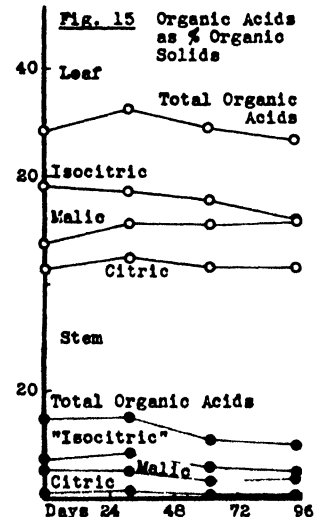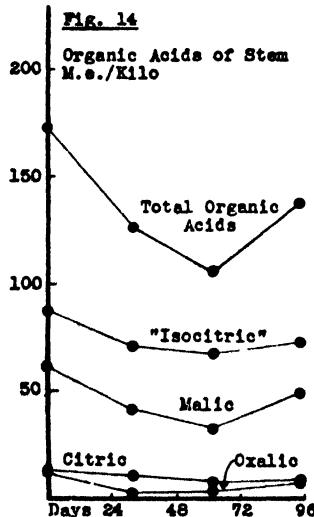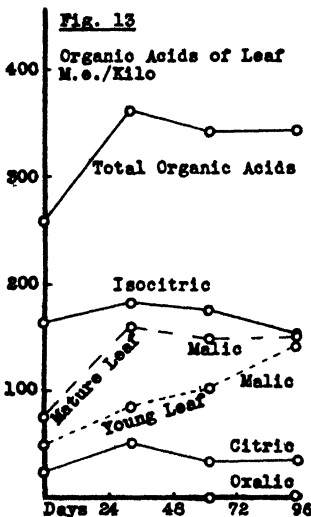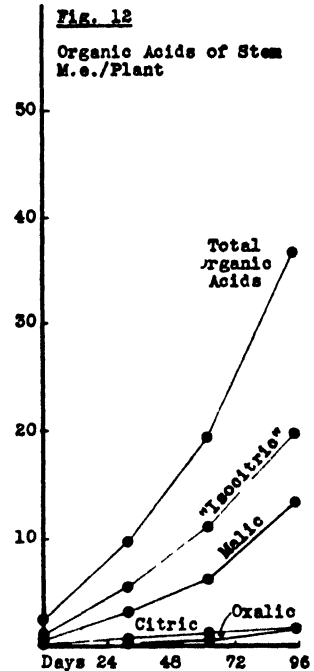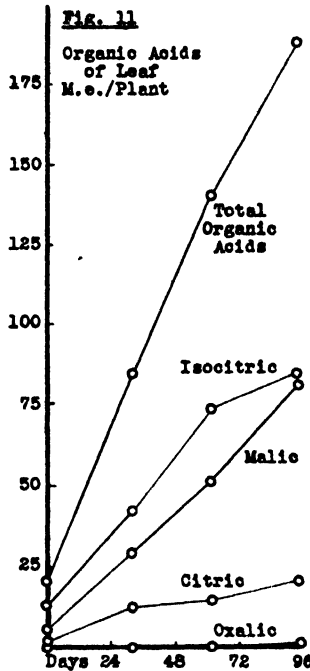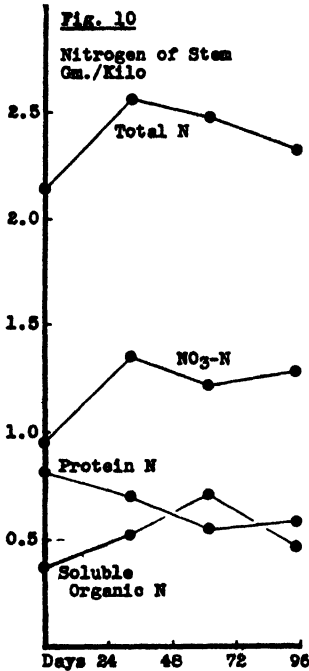
Some discussion is necessary of the meaning of the term "isocitric acid" as used in the analyses of these tissues. It has been shown that by far the greater part of the difference between the total organic acids and the sum of the malic, citric, and oxalic acids in Bryophyllum leaves consists of isocitric acid (10). Accordingly, this difference, which in previous papers from this laboratory is denoted as "unknown organic acid," may, in the

particular case of the leaves of Bryophyllum, be used as an approximate measure of the isocitric acid content. The error with this tissue is certainly not important. But we have obtained, as yet, no direct evidence that isocitric acid is present in the stems of this plant. It seems a reasonable assumption, however, that the qualitative composition of the stem will not differ greatly from that of the leaf and, accordingly, the proportion of unknown organic acids in the stem has been provisionally denoted as "isocitric acid." Analytical methods for isocitric acid have been developed during the past two years in other laboratories (5, 8). These methods, however, depend on the use of specific enzymes, and their application to the analysis of plant tissues has not yet been made in this laboratory.

Figure 13 shows the organic acids of the leaf tissue expressed in concentration units. During the first 32 days of growth, there was a marked increase in the concentration of the total acids much of which arose from the increase in malic acid; subsequently the concentration diminished moderately. The behavior of malic acid in the mature and in the young leaves was different. The young leaves showed a steady increase in malic acid throughout the experimental period, but the mature leaves increased in malic acid only in the first interval between observations.

The behavior of the concentration of the acids in the stem tissue is shown in figure 14, which is plotted on a scale twice that of figure 13. The total acids decreased for the first 62 days and then increased, and both malic acid and isocitric acid shared in the change. The small concentrations of citric and oxalic acids present altered very little.

Figure 15 shows the organic acids of both leaf and stem tissue as percentages of the organic solids. Isocitric acid is the predominant organic acid and was present to the extent of 18% of the organic solids of the leaves of the youngest plants but the proportion decreased as the plants matured. Young plants are thus obviously to be preferred for the preparation of isocitric acid in quantity. With such material, the relative proportion of isocitric to citric acid is greatest, a circumstance favorable for the separation by chemical means, and greater yields would be anticipated. It would also be more economical of time to work with young plants.

The data for the concentration of the acids in the organic solids of the stem are plotted in the lower part of figure 15. As has already been pointed out, this tissue is relatively low in organic acids and the acidity diminishes with increasing age. The stems of the young plants contained 15% of the organic solids as organic acids and this proportion dropped to 10%. "Isocitric acid" made up about 8% of the solids of the young stems and 5% of the oldest. The proportion of malic acid was somewhat less in each case.

Growth in terms of carbohydrates

Figure 16 shows the rate of increase in total and unfermentable carbohydrates in the leaf tissue, the data representing the reduction measured with the Shaeffer-Somogyi sugar reagent, and being expressed in terms of

glucose. The leaf tissue is moderately rich in sugar, the composition, expressed as percentage of the organic solids, ranging from 8.6% to 6.8% in the mature leaves, and from 3.7% to 4.9% in the young leaves. The present figures represent, of course, only the condition at the time of collection of the samples with respect to a component that fluctuates within fairly broad limits during the day. However, the rate of accumulation of total carbohydrate was essentially linear over the period of observation, suggesting that the samples were taken at comparable stages in this metabolic process.

The component designated unfermentable carbohydrate represents a titration for sugar carried out after treatment of the extract from the leaf with yeast. Clearly, a substantial part of the total carbohydrate consists of substances that are not removed by this treatment, and it is well known that components of similar behavior are commonly found in leaf tissues. The rate at which this type of carbohydrate accumulated seemed to increase moderately as the plants developed.

The quantities of crude fiber, determined by the conventional analytical method, are also shown in figure 16. This material doubtless consists mainly of cellulose and the rate of accumulation serves as an approximate measure of the rate of growth of the cell walls. The curve is, within the limits of error, linear throughout the period of study. It is of interest that the quantities of crude fiber in the leaf tissue are nearly identical with the quantities of soluble carbohydrates in each sample. However, no significance should be attached to the coincidence of the curves. No such agreement was observed in the case of tobacco leaves; in these, the slopes of the analogous curves were quite different.

The chief carbohydrate component of the leaf tissue was starch. The analytical values have been divided by 5 in order to bring them within the scale of figure 16, and it is to be noted that the rate of accumulation of starch was continually accelerated as the plants matured. By far the greater part of the starch was found in the older leaves and it is clear that the rate of growth, as measured by the accumulation of starch, is a function quite different from the rates as measured, for example, by the organic solids or the protein nitrogen, both of which were essentially linear. As the plants increased in age, the leaves became increasingly efficient with respect to the synthesis, or at least the storage, of starch.

The data plotted in figure 17 shows that the stem is, in general, lower in carbohydrates than the leaves. The values for total carbohydrate range from 3% to 5% of the organic solids. Unfermentable carbohydrate was present although in relatively small proportion. The rate of accumulation was slow at first but then followed an approximately straight line curve.

The most important carbohydrate component of the stem tissue was cellulose as is shown by the curve for the crude fiber. The rate of deposition of cellulose was slow at first, but proceeded subsequently at a linear and more rapid rate. However, there was less crude fiber in the stem than in the leaves throughout the period of observation. This is in marked contrast to the

behavior of the tobacco plant, the woody stem of which soon exceeds the leaves in quantity of crude fiber present and, at maturity, contains ten or more times as much.

Fig. 16 Carbohydrates of Leaf Gm./Plant

Fig. 17 Carbohydrates of Stem Gm./Plant

Fig. 18 Carbohydrates Gm./Kilo

Fig. 19 Protein and Organic Solids Tobacco and Bryophyllum Leaf Gm./Plant

Fig. 20 Organic Acids Tobacco and Bryophyllum Leaf M.e./Plant

Fig. 21 Carbohydrates Tobacco and Bryophyllum Leaf Gm./Plant
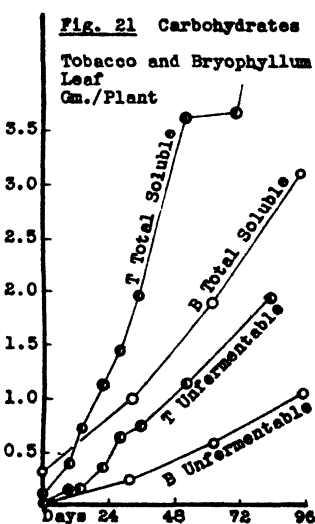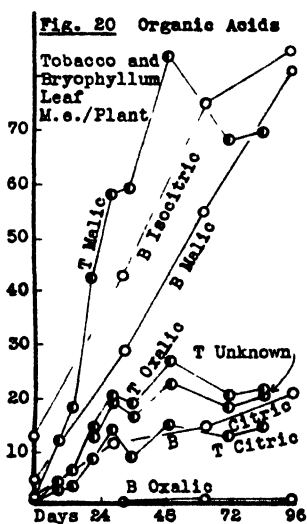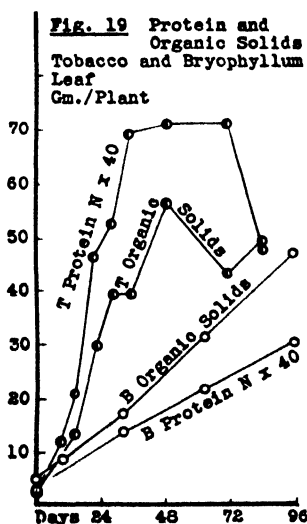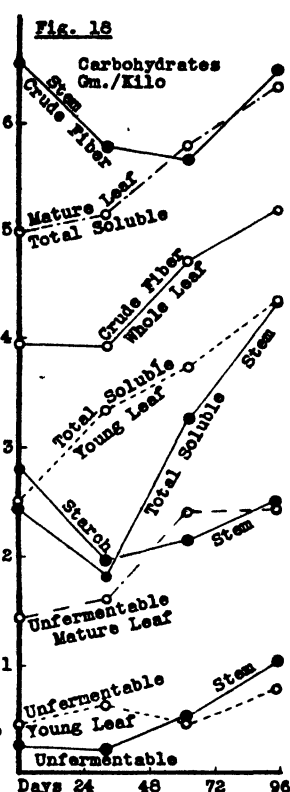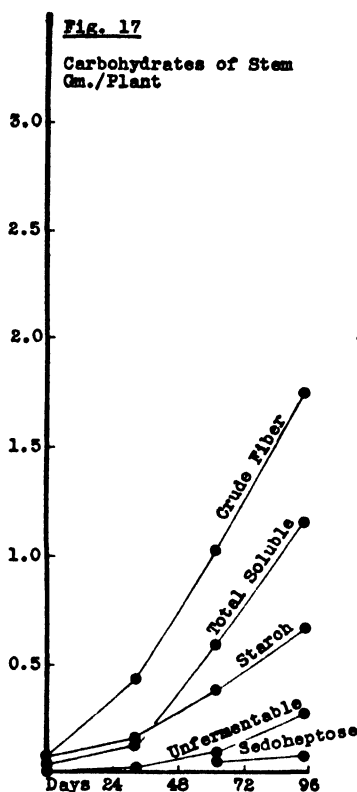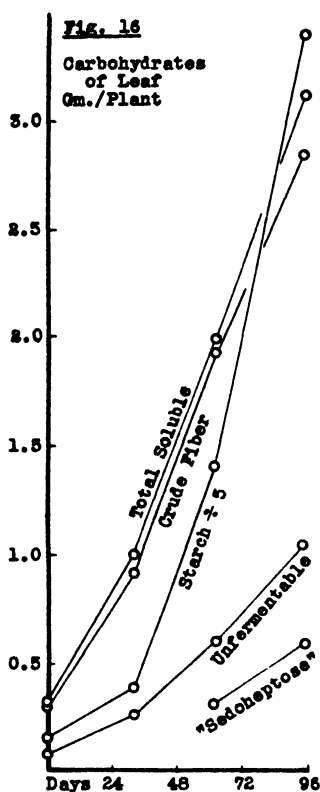
The stems contained only moderate quantities of starch, less indeed than of soluble carbohydrates, and the rate at which starch appeared conforms with the rates for the soluble carbohydrates. There would appear to be little connection, therefore, between starch synthesis in the stem and that in the leaves.

The concentration of the carbohydrate components in terms of fresh weight is shown in figure 18. In the mature leaves, the total carbohydrates increased moderately with age, but, in the young leaves, the rate of increase was somewhat greater. However, the concentration was invariably much higher in the mature than in the young leaves. The unfermentable carbohydrate components were also present in higher concentration in the mature than in the young leaves; there was a moderate increase in the mature leaves with age, but the concentration in the young leaves remained fairly constant throughout.

The concentration of total carbohydrate in the stem dropped sharply during the first 32 days, but subsequently increased to approximately twice the minimal value.

The concentration of crude fiber in the entire leaf tissue is also shown in figure 18; there was no change during the first 32 days of growth but, subsequently, the proportion increased. The behavior of the crude fiber in the stem tissue was quite different; it decreased in relative proportion at first, remained nearly constant for a considerable period and then increased. The concentration changes of fiber in the stem were thus analogous to those of the total organic solids (fig. 4) and the total organic acids (fig. 14).

The concentration of the starch in the leaves has not been plotted in figure 18 since the values are outside the scale of this diagram. In the young leaves it ranged from 8.8 grams per kilo in the plants at zero time to 17.0 grams per kilo in those 95 days later. The corresponding figures for the mature leaves were 11.1 and 24.7 grams per kilo. The values for the stem are shown, however, and indicate that the stems were relatively richer in starch at zero time than they later became. The changes in concentration conform moderately well with those of the total carbohydrate in the same tissue.

Sedoheptose

In figures 16 and 17, an additional component of the soluble carbohydrate fraction is shown at the bottom of the diagram and is marked "sedoheptose." Sedoheptose was discovered by LAFORGE and HUDSON (6) in 1917 in the leaves of *Sedum spectabile*. It is characterized by the fact that the reducing power of a solution of the sugar is diminished by approximately 80% of its original value when the solution is heated with 1% hydrochloric acid. The change is the result of the loss of one molecule of water which gives rise to the formation of an anhydride or sedosan; the reaction appears to be an equilibrium. BENNET-CLARK (1) has employed a method based on this change in reducing power on treatment with acid to detect sedoheptose in the leaves of succulent plants, among them *Bryophyllum calycinum*, and has further noted, in the case of *Sedum praealtum*, that the sugar component which has this property undergoes wide diurnal variations in amount, these variations being reciprocal with those of the acidity. WOLF (16), however, was unable to confirm this in the case of *Bryophyllum calycinum;* rather, he demonstrated reciprocal variation of the fermentable carbohydrates and

of the starch as against the changes in the acidity.   Nevertheless, he secured evidence of the presence of sedoheptose in Bryophyllum leaves by the isolation of a phenylosazone that corresponded closely in properties to that described by LaForge and Hudson.

A brief study of the reducing power of extracts from Bryophyllum leaves confirmed the presence of a carbohydrate which was diminished in reducing power when the extract was boiled with dilute acid.   These analyses were carried out on extracts that had not been treated with yeast; otherwise lower and erratic results were obtained.   Experiments in which sulphuric acid of concentrations in the range 0.2 to 1.0 N were used showed that a concentration of 0.35 N was most satisfactory for the formation of the anhydride ring. With this reagent, a maximal diminution of the reducing power was secured after the solution had been boiled for 30 minutes.   No further loss occurred after the solution had been boiled for 30 additional minutes.   Weaker acid reagents required longer times while a reagent as strong as 1.0 N gave smaller diminutions probably because of the hydrolysis of complex carbohydrates.

The diminution of the reducing power, expressed as glucose, is plotted in figure 16 as "sedoheptose" in spite of the fact that it is known that the anhydride formation with pure sedoheptose is incomplete.   LaForge and Hudson state that the equilibrium occurs when 80% of the sugar has been converted, while Bennet-Clark observed 75%.   Furthermore, the relationship between the sugar reagent standardized in terms of glucose and the quantity of sedoheptose reduced is unknown.   Because of these two uncertainties, it seemed best to plot the actual quantities of reagent reduced, in terms of glucose, and to postpone the interpretation in terms of the quantities of sedoheptose involved.

With these restrictions of the meaning of the term defined, "sedoheptose" was observed in significant amounts only in the mature leaves of the 62- and 95-day plants.   In these, it made up a substantial part of the unfermentable carbohydrate, almost certainly a larger part than is suggested by the relative positions of the curves drawn in the figures.   The more mature leaves of the youngest plants gave evidence of only a trace of this component, while the young leaves of all save the 95-day plants gave either an increase in reduction when the extracts were heated with acid or no change.   The stems of the 62- and 95-day plants also showed evidence of a trace of "sedoheptose" but, if any were present in the stems of the 0- and 32-day plants, it was not detected since the reduction was increased slightly by the treatment with acid.   Clearly, therefore, "sedoheptose" is a component that could be demonstrated only in the mature leaves of fairly old plants and, possibly, in the stems of the same plants.

Composition of Bryophyllum tissues

Table II shows estimates of the quantitative composition of the leaf and stem tissue of these samples of Bryophyllum plants.   The data for protein

TABLE II

Composition of Bryophyllum Plants

Figures not otherwise designated are grams per single plant.

	LEAF				STEM			
Growth period (days)	0	32	62	95	0	32	62	95
Protein (N × 6.25)	0.619	2.181	3.456	4.756	0.075	0.337	0.625	0.994
Soluble nitrogenous components (N × 10)	0.150	0.780	2.13	1.62	0.050	0.390	1.29	1.22
Starch	0.810	2.07	7.02	12.15	0.040	0.153	0.393	0.668
Crude fiber	0.310	0.913	1.94	2.85	0.093	0.447	1.03	1.75
Ether extract	0.132	0.534	0.992	1.48	0.015	0.068	0.147	0.373
Soluble carbohydrate	0.331	1.003	1.970	3.116	0.035	0.140	0.598	1.165
Isocitric acid	0.828	2.736	4.685	5.398	0.079	0.349	0.707	1.272
Malic acid	0.361	1.980	3.465	5.466	0.057	0.213	0.408	0.886
Citric acid	0.128	0.777	0.930	1.323	0.012	0.055	0.080	0.120
Oxalic acid	0.002	0.006	0.033	0.063	0.007	0.014	0.034	0.082
Total known organic solids (estimated)	3.671	12.98	26.62	38.22	0.463	2.166	5.312	8.530
Total organic solids (determined)	4.80	17.25	31.83	47.44	1.02	4.90	11.82	23.55
Unknown organic solids	1.18	4.27	5.21	9.22	0.557	2.73	6.51	15.02
Unknown organic solids as percentage of total organic solids	24.6	24.7	16.4	19.4	54.6	55.7	55.1	63.8
Inorganic solids	0.84	2.66	4.77	6.01	0.196	0.907	2.075	3.10

are computed from the protein nitrogen by multiplication by the conventional factor 6.25, a procedure that is admissible in the absence of information on the actual nitrogen content of the tissue protein. The estimate of the quantities of soluble nitrogenous components is made by multiplying the data for non-protein soluble nitrogen, exclusive of nitrate, by the factor 10. This is doubtless an overestimate since the average nitrogen content of the substances in this group, presumably amino acids, amides, polypeptides, and basic substances such as choline, purines, and basic amino acids is greater ·than 10%. However, it has been found, in other samples of Bryophyllum leaves, that from 35% to 40% of the soluble nitrogen is normally present as amino nitrogen; thus a considerable part of the substances concerned probably does consist of amino acids and the factor 10 can be used for these without grave error.

Table II also gives data for the ether extract of the samples. This was obtained by the conventional procedure and represents chlorophyll, lipid pigments, and other fat-solvent soluble material; probably only a small part of it consists of true fat.

The quantities of organic acids were computed from the analyses in terms of milliequivalents by the use of the appropriate factors.

The sums of the estimates are shown, together with the determined quantities of organic solids; the difference between these thus represents that part of the organic material concerning which no qualitative information is available. In the leaf tissue, this unknown part amounts to from one-quarter to one-sixth of the organic solids. In the stem tissue, on the other hand, the unknown part makes up appreciably more than one-half of the total, and suggests that a number of major components await detection and determination. Nothing but speculation can be offered concerning these unknown components in either leaf or stem tissues. However, such substances as glucuronides of the pectin type are probably present in the leaf and complex carbohydrates such as hemicellulose in the stem. Clearly, much remains to be learned regarding the qualitative composition of the tissues of this plant.

Comparison of Bryophyllum with tobacco

In figures 19, 20, and 21 are shown data taken from a study of the rate of growth of the tobacco plant (13) plotted together with a few of the more important items of the present data on the Bryophyllum plant in order to afford a comparison of the behavior of the two species. Figure 19 shows the organic solids and the protein nitrogen of the leaf tissues, these being selected as perhaps the most clearly illustrative of the relative rates of growth. The protein nitrogen values have been multiplied by 40 in order to obtain curves that can be plotted on the same diagram with the solids. Zero time for the tobacco plants is arbitrarily taken as the point at which the composition with respect to nitrogenous components was essentially identical with that of the youngest Bryophyllum plants. This occurred 26 days after the tobacco seedlings had been set in the field when the plants had become well established

and were just beginning to grow at a rate which followed a nearly linear course for the next four or five weeks. It is assumed that, during this period, the plants were passing through a phase of growth analogous to that of the present series of Bryophyllum plants. At all events, this was the purely vegetative stage in the growth period of the tobacco plants. The first samples of inflorescence tissue, mainly flower buds, were collected from the tobacco plants at 61 days from transplanting (*i.e.*, at 35 days on the scale of figures 19 to 21) and subsequent samples contained increasing quantities of flowers and developing seed pods. The change in the course of the metabolism, as the plants passed into the reproductive phase, is illustrated on the curve for the leaf protein which flattened out and then fell as protein was withdrawn from the leaves during the ripening of the seeds. There was also a loss of organic solids from the leaves. The main point of interest in the present comparison is, however, the contrast between the relative rates at which leaf protein and leaf organic solids were formed during the first 40 days of growth of the tobacco plant and their behavior in the Bryophyllum plant. Clearly, the tobacco leaf is considerably richer in protein, in relation to the organic solids, than is the Bryophyllum leaf, while the rate of growth, as measured by the rate of accumulation of the leaf protein, is about four times as rapid. The rate of growth, as measured by the organic solids of the leaf, is about twice as great.

Figure 20 shows the composition of the leaves of the two species with respect to organic acids. In the tobacco leaf, malic acid is by far the predominating acid component, and the curve follows in fairly close detail that for the organic solids in figure 19. Oxalic acid is next in relative importance as a component, and the "unknown" organic acids are present in closely similar amounts. The curve for the "unknown" acids of the tobacco leaf should be compared with that for isocitric acid in the Bryophyllum leaf, the main acid component of this species. Both curves depend upon analytical data obtained in the same way, the difference being that the "unknown" acids of the Bryophyllum leaf have been shown to be comprised largely of isocitric acid. Whether or not isocitric acid occurs in tobacco leaf has not been demonstrated. However, observations on the organic acid esters of high boiling point obtained from tobacco leaf tissue suggest that isocitric acid can be present only in traces if at all.

Citric acid is a minor component of both the tobacco leaf and the Bryophyllum leaf, and the curves for the rate of accumulation in the two species are closely similar; in fact this is the only point in which the two are closely alike.

The curve for malic acid in the Bryophyllum leaf represents the composition with respect to this component at noon on sunny days. Had the samples been collected earlier in the day, the curve would, doubtless, have been displaced vertically upwards and might also have had a somewhat different slope. Nevertheless, the rate of accumulation of malic acid in this plant, as a function of age and with this restriction on the time of collection, is clearly less than it is in the tobacco plant.

The curves for the total organic acidity of the two species are not plotted in figure 20; they show, however, that the tobacco plant accumulates organic acids in the leaves, during the approximately 40-day period of its maximal growth rate, somewhat more rapidly than the Bryophyllum plant. However, the check that is placed on the development of the tobacco leaf by the onset of the reproductive phase brings the synthesis of organic acids to a stop, while accumulation of acids in the Bryophyllum leaves continues as is shown in figure 11. Nevertheless, if observations on Bryophyllum leaves were made in the early morning, there is little doubt that the rate of accumulation of total acids would closely approach that of the tobacco leaf.

The qualitative composition and, with the exception of citric acid, the relative rates of synthesis of the organic acids in the two species are entirely unlike. The differences with respect to oxalic acid and isocitric acid are perhaps the most striking. Oxalic acid is an important component of tobacco leaf tissue but is present in Bryophyllum leaves only in traces. The reverse is true for isocitric acid. The metabolic systems that lead to the synthesis of organic acids in the two plants are thus entirely unlike; although there are, doubtless, certain features common to the two, since malic and citric acids are components of both, the details of the chemical mechanisms in which organic acids share are manifestly widely different.

Figure 21 shows the relative rates of accumulation of soluble carbohydrates in tobacco and Bryophyllum leaves. Although the type of tobacco plant described (the so-called Connecticut shade-grown tobacco) is characterized by a somewhat low level of carbohydrate content, particularly of starch, it is obviously a species that synthesizes soluble sugars in the leaves more rapidly and in larger quantities than the Bryophyllum plant. Much of the unfermentable carbohydrate of the mature Bryophyllum leaf is doubtless sedoheptose but this is certainly not true of the tobacco leaf, although unfermentable carbohydrates make up a large part of the soluble sugars in this species. Thus, both qualitatively and quantitatively, the composition of the two kinds of leaves is widely dissimilar.

Summary

The composition of the leaf and stem tissue of *Bryophyllum calycinum* plants, harvested at noon on sunny days at intervals over a period of 95 days, has been determined in order to obtain fundamental data upon the rate of growth of a plant which is characterized by a pronounced diurnal variation in organic acid content. The composition is recorded in terms of grams per plant. The rate of accumulation of the fresh weight and of many of the components, in particular the organic solids, the ash, the water, the protein, the nitrate nitrogen, the soluble carbohydrates, the crude fiber (cellulose), the total organic acids, and the malic acid followed essentially straight line curves throughout the period of observation. On the other hand, the total nitrogen followed a curve somewhat concave to the time axis, as was true also of the isocitric acid and citric acid, suggesting a gradual slowing of the rate

of accumulation of these components, while the starch of the leaves, by far the most plentiful known component of this tissue, followed a curve markedly convex to the time axis indicating an increase in relative capacity for the storage of starch as the leaves matured.

The data have also been computed in concentration units, namely in grams per kilo of fresh weight of the tissues. These curves show a moderate degree of irregularity in the relative concentrations of the various components at different stages of growth.

Comparison of the data with similar results for the tobacco plant, at an analogous period in the life cycle, showed marked differences in the rate of accumulation of most of the components. The tobacco plant grows much faster as measured by almost all of the criteria. The most important exception is in the rate of accumulation of organic acidity; isocitric acid is formed almost as rapidly by the Bryophyllum plant as malic acid is by the tobacco plant, and citric acid is formed at almost equal rates in the two species. On the other hand, oxalic acid, which is a major organic acid component of the tobacco plant, is present only in traces in Bryophyllum.

Isocitric acid is the predominating organic acid component of Bryophyllum leaf tissue and is present in young leaves to the extent of about 18% of the organic solids; such tissue is accordingly valuable for the preparation of this rare acid in quantity.

We are indebted to the DEPARTMENT OF ANALYTICAL CHEMISTRY of this Station for the determinations of crude fiber and ether extract in these samples.

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SOME MOLD–INDUCED CHANGES IN SHELLED CORN[1]

C. M. NAGEL AND G. SEMENIUK

Introduction

Shelled corn which molded during storage was found to be high in fat acidity and moisture percentage and low in bushel weight (10). Because many different fungi were associated with the molded condition of the corn, the extent to which the individual fungi contributed to these changes was of interest. Laboratory studies were conducted by infesting sterile corn with pure cultures of different fungi which were obtained from the naturally molded shelled corn found in the storage bins (10). Determinations were made on the laboratory-molded corn for changes in organic matter, water-soluble and -insoluble nitrogen, fat acidity, pH and water content. These same fungi were also cultured on Czapek-Dox medium to determine their carbohydrate-utilizing and acid-producing capabilities on this medium in comparison with corn.

Materials and methods

The 9 fungi used were: *Aspergillus flavus* Link, *A. candidus* Link, *A. niger* van Tieghem, *A. amstelodami* (Mangin) Thom and Church, *Penicillium palitans* Westling, *P. chrysogenum* Thom, and cultures I and II *P. rugulosum* Thom and *Mucor racemosus* Fresenius (these cultures differed in color and in growth rates on agar media). Subcultures of each of these were prepared in quadruplicate on sterile corn as follows: Lots of 150 gm of Iowa hybrid 939 corn with approximately 15% moisture were placed in each of a number of 500-ml. Erlenmeyer flasks which were plugged with cotton and steamed at 15 lbs. pressure for 45 minutes on two consecutive days. After the final steaming, and while the flasks and corn were still hot, measured quantities of hot, sterilized, distilled water were added aseptically to the corn to bring it to approximately 32% moisture content. Several days at room temperature were then allowed for the corn to absorb the added moisture. This process was facilitated by frequently shaking and rotating the corn within each flask. The corn was then carefully infested with dry spores of each of the fungi except *Mucor racemosus*. The spores of the other fungi were taken from old sporulating cultures on the flattened end of a nichrome transfer-needle and carried to the mouth of the proper flask at which position they were showered. The spores of *M. racemosus* were prepared in aqueous suspension and distributed by drops in amounts of 1 ml. per flask. The con-

tents of each flask were then mixed by hand-rotating the flask for about 1 minute and this was repeated several times over the succeeding days. The flasks of corn were then set aside in the laboratory for periods up to 4 weeks for the fungi to grow. Controls without mold infestation were also included in this test.

At the different analysis periods, the moldy corn and the mold-free controls were emptied on pieces of cheesecloth from each of the quadruplicate flasks and dried for several days at the temperature of the laboratory with the aid of an electric fan. Further drying was done at 70° C. over a 4–5-day period in an electrically heated oven, after which the material was ground for analysis to pass a 200-mesh sieve. The moisture content of the ground material was determined with a vacuum oven at 70° C. Fat acidities were determined by the method of ZELENY and COLEMAN (14). Water-soluble nitrogen was determined on 100 ml. of aqueous extract from 2 gm. of the material (water-free basis). This extract was prepared by shaking the material in water for 8 hours at the temperature of the laboratory. Water-insoluble nitrogen was taken as the difference between the total nitrogen in the unextracted moldy corn and its water-soluble nitrogen content. Determinations of pH were made with a Coleman electrometer on a suspension of 1.0 gm. ground material in 10 ml. of boiled, distilled water. The water content of each culture was taken as the difference between the wet and dry weights of the culture flask contents. The organic matter content of each culture was taken as the dry matter content of each flask.

Liquid culture studies of the fungi were conducted on Czapek-Dox medium. Twenty-five milliliters of this medium were placed in a number of 125-ml. Erlenmeyer flasks and sterilized at 15 lbs. steam pressure for 15 minutes. Spores of the different fungi were introduced onto this medium in a manner similar to that on corn, except that in the case of *M. racemosus* only two drops of a spore suspension were added to the medium in each flask. The fungi were allowed to grow on this medium for 1, 2, and 4 weeks at ordinary laboratory temperatures. At these periods the developed mycelium was removed from a number of cultures by decantation and filtration through pieces of washed percale cloth after which it was carefully removed from the cloth with forceps, washed in small quantities of hot, distilled water, dried at 70° C. for 3 days, and weighed in a closed weighing bottle. The filtrates from the liquid culture were increased to 100 ml. volume with distilled water, then analyzed for the remaining glucose and for other reducible substances by means of the copper method of VAN DER PLANK (13) and the iodometric method of HINTON and MACARA (5). The titrable acidity and pH of this diluted filtrate were also determined.

Experimental results

GROWTH OF THE FUNGI ON CORN

Two trials were conducted on corn to determine the organic matter, fat acidity and nitrogen changes resulting from the activity of the 9 fungi.

The first trial was divided into two parts with 4 of the 9 fungi being compared at one time and the remaining five at another time. The second trial compared all of the 9 fungi simultaneously. In the first trial, organic, matter, fat acidity, water-soluble, and -insoluble nitrogen were determined, while in the second trial water formation and pH of the molded corn were also determined. Because the second trial yielded essentially the same results as the first trial and simultaneously compared the 9 fungi and afforded the information on water formation and pH changes, its data are mainly considered here. Some pertinent data of the first trial are also presented.

ORGANIC MATTER LOSSES

As may be seen from the data of the second trial shown in table I, four of the nine fungi induced marked organic matter losses. The greatest loss (approximately 40%) was induced by *Aspergillus flavus*, *A. niger*, *Penicillium chrysogenum* I, and *P. chrysogenum* II in the 4-week interval following infestation. Losses of 20.1%, 14.5%, 11.9%, 10.4%, and 6.4% in this same interval were induced by *Aspergillus candidus*, *Penicillium palitans*, *P. rugulosum*, *Mucor racemosus* and *Aspergillus amstelodami*, respectively. The losses at the 2-week interval with 6 of the fungi were approximately one-half of these while those with *Penicillium palitans*, *P. rugulosum*, and *M. racemosus* were lower.

In the first trial, where the analyses were made at weekly intervals instead of at 2-weekly intervals as in the second trial, similar losses of organic matter were obtained. These losses were approximately linear with all the fungi over the 4-week period following inoculation. As in the second trial, *Aspergillus flavus*, *Penicillium chrysogenum* I, *P. chrysogenum* II, and *Aspergillus niger* were similar in their high rate of organic matter decomposition (fig. 1), causing approximately 40% losses of organic matter in 4 weeks, even though the first two and the last two fungi were tested at different times. The other fungi, which were also tested at different times, comprised a group with organic matter decomposition at approximately one-fourth of this rate.

FAT ACIDITY CHANGES

The high fat acidities of around 200 units and as high as 380 units presented in table I and figure 1 for the two trials compared favorably with the high fat acidities found occurring in molded shelled corn under commercial storage (10). The highest value in this instance of 273 units per 100 grams of dry matter was greater than any reported by ZELENY (14) for sample grade corn, but approached that for badly heated or *Diplodia zeae* infected corn. In the present experiments, 200 and more units of fat acidity were produced by *Aspergillus flavus*, *A. niger*, *Penicillium chrysogenum* I and *P. chrysogenum* II, the fungi which caused the greatest and most rapid organic matter losses and by *Aspergillus amstelodami* and *Mucor racemosus*, the fungi which caused lower organic matter losses. The remaining 3 fungi,

Penicillium palitans, P. rugulosum, and *Aspergillus candidus* produced fat acidity values of between 100 and 150 units (table I and fig. 1). These values were low in comparison with those produced by the other fungi, but approached the fat acidities found in the poorest of sample grade corn (**14**).
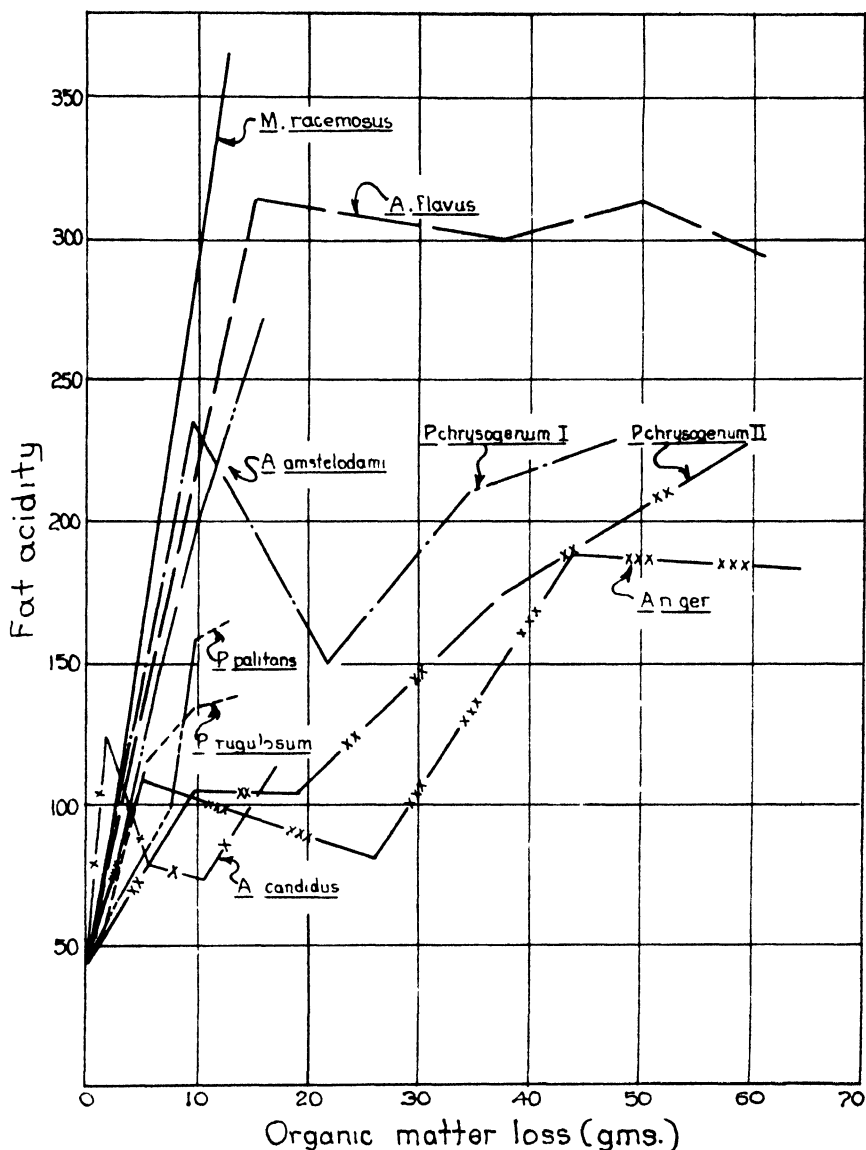
FIG. 1. Relation between organic matter loss and fat acidity at 4 successive weekly intervals. Data from the first trial.

As may be seen from the data in table I, the maximum fat acidity values produced by the different fungi were reached by approximately the end of the 2-week period, except for *Mucor racemosus*, which produced an even greater fat acidity by the end of the 4-week period. In the first trial, on the other hand, the maximum fat acidity values were reached within the first week by *Aspergillus flavus, A. candidus,* and *Penicillium chrysogenum*

TABLE I

ANALYSIS OF STEAM STERILIZED CORN MOLDED BY NINE FUNGI

Fungus	Weeks of Develop-ment	Organic Matter Loss (gm.)	Organic Matter Loss (%)	Moisture Formed (gm.)	Moisture Content (Wet Basis) (%)	Fat* Acidity (units)	pH	Nitrogen* Water-Soluble (gm.)	Nitrogen* Water-Insoluble (gm.)	Nitrogen* Total (gm.)
None (control)	2	0†	0	0	32.7	43.9	6.0	0.17	1.96	2.13
	4	0†	0	0	32.8	43.3	5.9	0.16	2.00	2.16
A. flavus	2	36.2	26.4	24.6	47.5	187.9	5.7	0.48	1.39	1.88
	4	60.0	44.0	34.8	57.0	176.9	5.2	0.41	1.21	1.62
A. candidus	2	17.6	12.8	11.4	39.5	81.3	6.1	0.41	1.60	2.01
	4	27.5	20.1	12.8	42.2	74.8	5.8	0.39	1.57	1.96
A. amstelodami	2	5.0	3.6	4.8	35.1	384.1	5.9	0.15	1.86	2.01
	4	8.7	6.4	3.5	35.4	247.9	5.8	0.18	1.82	1.98
A. niger	2	28.8	21.0	23.4	45.4	180.5	3.4	0.53	1.40	1.93
	4	52.7	38.6	32.7	54.2	129.0	4.1	0.33	1.45	1.78
P. chrysogenum I	2	26.3	19.1	20.6	44.0	181.4	4.7	0.39	1.63	2.02
	4	53.1	38.9	31.2	54.0	136.4	5.1	0.40	1.47	1.87
P. chrysogenum II	2	27.3	19.8	19.7	44.0	252.8	4.9	0.74	1.34	2.08
	4	56.7	41.6	30.9	55.0	209.6	5.1	0.41	1.38	1.79
P. palitans	2	5.5	4.0	5.1	35.3	113.0	5.1	0.20	1.87	2.07
	4	19.7	14.5	10.8	39.9	120.6	5.4	0.33	1.75	2.08
P. rugulosum	2	5.2	3.8	3.1	34.6	104.9	5.5	0.26	1.87	2.13
	4	16.2	11.9	7.4	38.1	99.4	5.2	0.31	1.82	2.13
Mucor racemosus	2	2.2	1.6	2.7	34.0	286.2	5.8	0.30	1.81	2.11
	4	14.1	10.4	8.6	38.1	312.6	5.1	0.37	1.80	2.17

* On the individual culture basis.
† Dry matter content of individual control flasks at 2 weeks = 137.2 gm.; at 4 weeks = 136.5 gm.

I, within the third week by *P. rugulosum, P. palitans* and *Aspergillus niger* and beyond the fourth week by *A. amstelodami, Mucor racemosus* and possibly *P. chrysogenum* II (fig. 1). In both trials, no over-all relationship was evident between the quantity of organic matter lost and the fat acidities produced. The fungi continued to decompose organic matter even after the maximum fat acidities were produced (table I and fig. 1).

pH CHANGES

The pH changes of the molding corn were in the acid direction (table I). The greatest drop in pH (from the initial 6.0 to 3.4) was induced by *Aspergillus niger*. No relationship was evident with the different fungi between the changes in pH, the fat acidities and the quantity of organic matter decomposed.

NITROGEN CHANGES

The changes in the nitrogen content of the corn (table I) reflected the metabolic activities of the fungi. In the two trials, the water-insoluble nitrogen decreased while the water-soluble nitrogen increased. Total nitrogen decreased, presumably because ammonia was formed and escaped as a gas. Only 8% of the total nitrogen in the corn was initially water-soluble, while the remainder was water-insoluble.

After 2 weeks of fungus growth (table I), the water-soluble nitrogen proportion in the moldy corn was changed to 25.5%, 20.4%, 27.5%, 19.3%, and 35.6% by *Aspergillus flavus, A. candidus, A. niger, Penicillium chrysogenum* I and *P. chrysogenum* II, respectively, and to 9.7%, 12.2%, and 14.2% by *P. palitans, P. rugulosum* and *Mucor racemosus*, respectively. The first 5 fungi caused the greatest quantity of organic matter loss in this interval; namely, 26.4%, 12.8%, 21.0%, 19.1%, and 19.8% respectively; while the last 3 fungi were very weak in this capacity, causing 4.0%, 1.8%, and 1.6% losses, respectively. *Aspergillus amstelodami* caused a 3.6% loss of organic matter without increasing the proportion of water-soluble nitrogen in the moldy corn over the original. However, since there was a lowering of water-insoluble nitrogen in the corn, this indicated that some nitrogen was being mobilized by this fungus.

After 4 weeks of fungus growth the proportion of water-soluble nitrogen in the moldy corn was further increased only by those fungi which caused but slight organic matter losses at the 2-week period. With those fungi that were the greatest decomposers of organic matter the water-soluble nitrogen did not undergo any further increase but even decreased in amount. Autolytic processes (**2, 3, 6**) presumably were responsible for this latter result.

RELATION BETWEEN NITROGEN CHANGES AND ORGANIC MATTER LOSSES

HECK (4) has pointed out that 40% to 70% of fungus nitrogen is water-soluble. In view of this finding, and those just presented, the extent of the decreases in the water-insoluble nitrogen and the increases in the water-soluble nitrogen obtained in the molding corn might be considered

as indicators of the relative extent of fungus development. In figure 2 is presented the linear relationship between the quantitative loss of organic matter by the different fungi after 2 and 4 weeks' development and the decrease in the water-insoluble nitrogen. Linearity was also obtained, as shown in figure 3, between loss of organic matter and the increase in the amount of water-soluble nitrogen at the 2-week period. At the 4-week period, this linearity was not obtained presumably because of autolytic
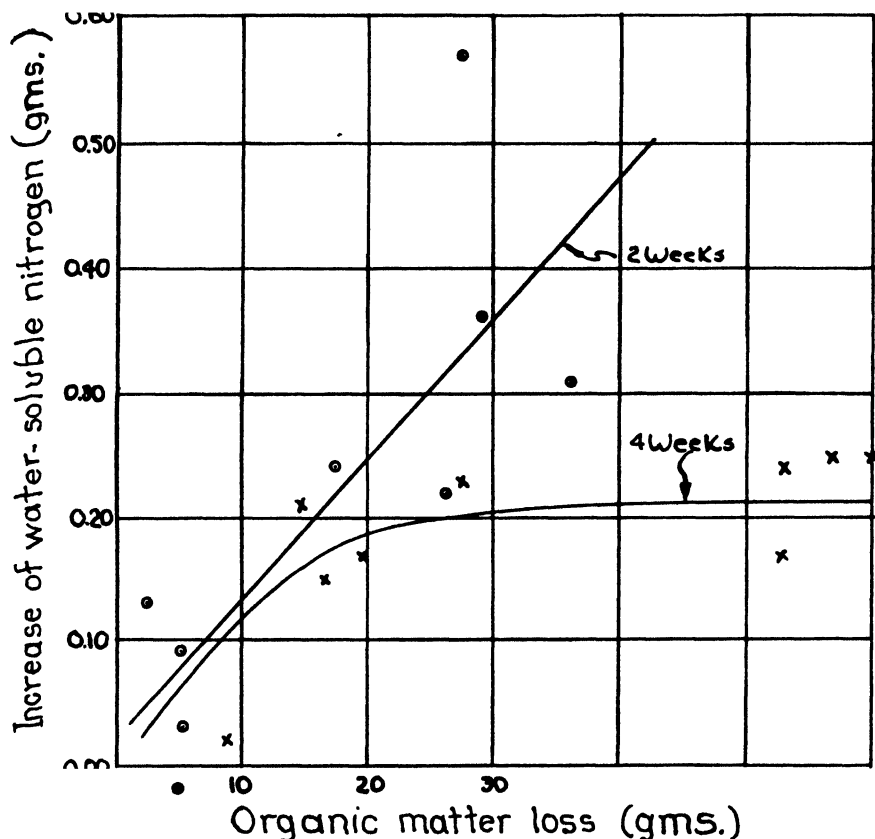
Fig. 2. Regression of increase in water-soluble nitrogen on organic matter loss after 2 and 4 weeks of fungus development. (Data from table I.)

processes surrounding the activities of *Aspergillus flavus*, *A. candidus*, *A. niger*, *Penicillium chrysogenum* I and *P. chrysogenum* II. Autolysis might be expected at this later period with these fungi because they were the most rapid growers and decomposers of organic matter. Continuation of growth activity eventually would result in the hyphae being in an unfavorable environment of depleted carbohydrate supply; in consequence they would undergo autolysis. This process in molds is known to result in nitrogen losses from the hyphae in the form of ammonia (**2, 3, 6**).

WATER FORMATION

The quantity of water that each fungus produced during its activity on corn is presented in table I. No special effort was made during this experi-

ment to prevent the loss of water by evaporation from each culture other than to plug the opening of each flask with a tight wad of cotton. Since the moisture content of the uninfested control corn remained unchanged after 2 and 4 weeks' standing in the laboratory, this suggested little or no water loss by evaporation.
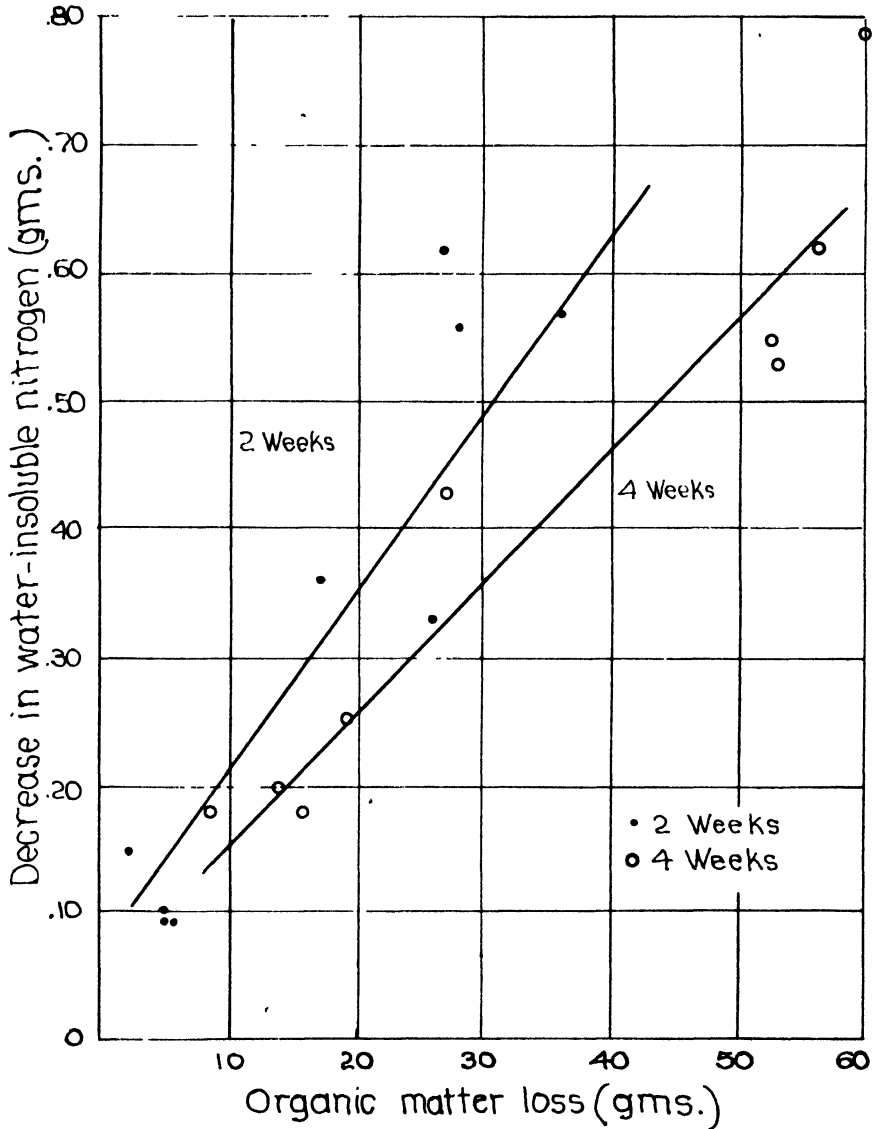
FIG. 3. Regression of decrease in water-insoluble nitrogen on organic matter loss after 2 and 4 weeks of fungus development. (Data from table I.)

The striking linear relationship between the percentage loss of organic matter induced by the different fungi after 2 and 4 weeks of development and the percentage of moisture (wet basis) in the molding corn at these two periods is presented in figure 4A. For every 1% loss of organic matter there was a corresponding rise of 0.55% moisture in the corn. The quanti-·tative relationship between these two variables after 2 and 4 weeks of fungus

development and during the interval between the second and fourth weeks of development is shown in figure 4B. The ratio between the organic matter lost and the water formed was 1 : 0.701 at the 2-week period, 1 : 0.601 at the 4-week period, and 1 : 0.380 during the interval between the second- and
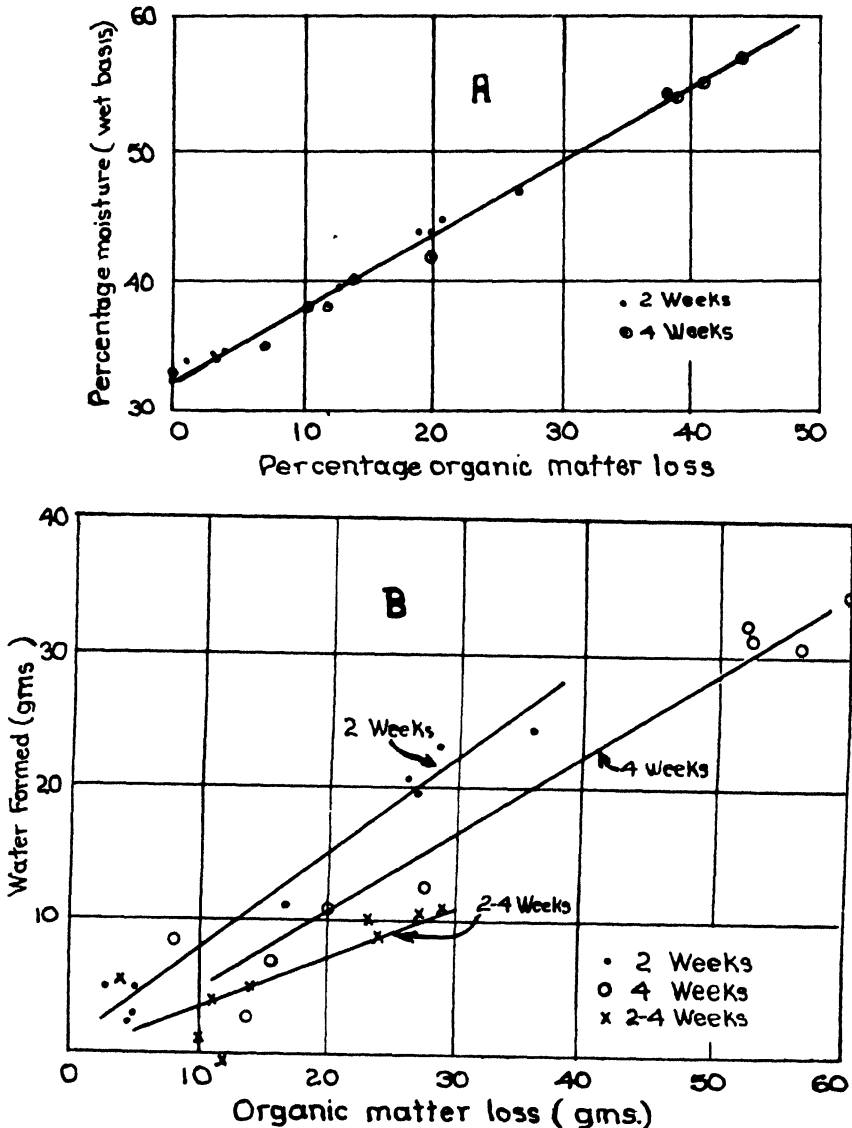
Fig. 4. A. Regression of percentage moisture (wet basis) on percentage loss of organic matter of corn digested by different fungi after 2 and 4 weeks. $Y = 0.55X + 32.63$. B. Regression of grams water formed by different fungi on grams organic matter loss after 2 weeks $(Y = 0.701X + 0.89)$, 4 weeks $(Y = 0.601X - 1.546)$ and during the 2 and 4 weeks' interval $(Y = 0.380X - 0.340)$.

fourth-week periods. One gram of starch on complete oxidation should yield, theoretically, 0.555 grams of water while fat should yield water at better than a 1 : 1 ratio. The ratio of 1 : 0.701 obtained at the 2-week period suggests utilization of both starch and fat by the fungi. At the beginning

of this study molds were observed to be developing in greatest abundance at the germ position on the kernels where the fats and oils are concentrated. The ratio of 1:0380 obtained during the interval between the second- and fourth-week periods would indicate the incomplete oxidation of organic matter if there was negligible loss of water through evaporation from the culture flask as suggested by the noninfested controls. The ratio of 1:0.601 obtained at the 4-week period would represent the average result of all these processes.

GROWTH OF THE FUNGI ON CZAPEK-DOX MEDIUM

The results of the experiments on corn reported above revealed considerable variation among the fungi in the quantity of organic matter decomposed and the fat acidity produced. To supplement these observations these same fungi were cultured on Czapek-Dox solution to determine whether or not their carbohydrate-utilizing and acid-producing powers on this medium would be similar to that on corn. The results for all the fungi except *Mucor racemosus* and *Aspergillus amstelodami*, which failed to develop in Czapek-Dox medium, are presented in table II.

In general, except for the differences in the nature of the two media and the different analytical methods used, the responses of the individual fungi were similar on the two media. *Aspergillus niger, Penicillium chrysogenum* I, and *P. chrysogenum* II produced a rapid lowering of the pH and a high titrable acidity on Czapek-Dox medium and on corn. *Aspergillus niger* gave essentially similar results on the two media except that comparatively low fat acidities were produced on corn. This latter result was probably because such benzene-insoluble acids as oxalic, citric, and gluconic (which are notable products of *A. niger* metabolism) were excluded from the determination. *Penicillium palitans* and *P. rugulosum* produced moderate titrable acidities on Czapek-Dox medium and on corn. The initial pH rise on Czapek-Dox medium was presumably due to the initially small acid-production and the liberation of the sodium ions from $NaNO_3$ by these fungi. Acidic substances were later produced, however, because the pH was lowered on prolonged growth of the fungus. These results were in general agreement with those on corn. *Aspergillus flavus* produced considerable quantities of kojic acid, which is a weak acid, on Czapek-Dox medium and on corn. The quantitative production of this acid (1) on Czapek-Dox medium may be deduced from table II by taking the difference between the "glucose" values determined by the copper and the iodometric methods (1). *Aspergillus candidus* produced negligible amounts of titrable acidity in Czapek-Dox medium and as a result the pH of the medium was thrown over to the alkaline side. Moderate fat acidities, however, were produced on corn, presumably because this fungus acted on the fat constituents of the germ.

The glucose utilization rates in Czapek-Dox medium were initially most rapid with *Penicillium chrysogenum* I, *P. chrysogenum* II, *Aspergillus niger*, and *A. flavus*, the 4 fungi most active in decomposing corn organic matter.

TABLE II

ANALYSES OF CULTURES OF 7 FUNGI DEVELOPING ON CZAPEK-DOX MEDIUM

| | Weight of Mycelium | | | pH of Medium* | | | Glucose Remaining† | | | | | | Titratable Acidity‡ |
| | | | | | | | Copper Method | | | Iodometric Method | | | |
	1 wk.	2 wk.	4 wk.	1 wk.	2 wk.	4 wk.	1 wk.	2 wk.	4 wk.	1 wk.	2 wk.	4 wk.	4 wk.
	mgm.	mgm.	mgm.				mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	
Penicillium palitans	94.4	124.5	149.0	6.09	5.28	4.25	871	638	235	867	624	277	6.40
P. rugulosum	94.2	152.4	189.3	5.55	4.39	3.86	870	542	105	854	528	129	6.16
P. chrysogenum I	69.6	132.0	176.3	3.66	3.33	3.84	650	121	6	660	153	53	10.00
P. chrysogenum II	99.7	173.4	190.2	3.62	3.30	3.68	504	42	6	555	54	35	8.92
Aspergillus flavus	145.7	144.0	138.7	5.70	5.21	5.71	712	630	464	705	832	876	7.68
A. niger	169.2	176.0	190.3	3.12	2.86	3.07	587	570	399	620	562	410	20.20
A. candidus	40.2	56.2	119.2	7.36	7.32	6.80	1026	922	628	1050	969	676	1.20

ermined on the culture filtrate diluted to 100 cc. Initial pH, value 4.26.
ial glucose supply was 1164 mgm.
liliters of 0.1 N NaOH to phenolphthalein per culture.

While these initial rates on Czapek-Dox medium were continued with the two cultures of *Penicillium chrysogenum* to the near complete utilization of sugar, the rates with *Aspergillus niger* and *A. flavus* were reduced markedly at the point of approximately 50% sugar utilization. Presumably, unfavorable changes occurred within the liquid medium supporting these latter two fungi so that their further activity was hindered. *Penicillium palitans, P. rugulosum*, and *Aspergillus candidus*, on the other hand, utilized sugar at a constant, slower rate over the 4-week culture period. These slower sugar utilization rates conformed to the slower rates of corn organic matter decomposition by these same fungi.

Discussion

The loss in organic matter and the changes in the moisture content and fat acidity of molding corn under pure culture conditions compared favorably with similar changes noted in molded corn found in storage bins under natural conditions (10). The high moistures and fat acidities observed in the molded corn in the bins (10) may be attributed in large part to the metabolic activities of the organisms themselves. The higher water-soluble nitrogen content of the molded corn observed in the laboratory reflected the activities of these organisms, and this would suggest that the higher amino acid content found in sample grade corn (corn having over 15% of damaged kernels) as against No. 1 to No. 5 yellow corn as noted by ZELENY and COLEMAN (14) may also be the product of fungus activity.

The effect of mold growth on the corn oil was not determined in the present study. WINTON, BURNET, and BORNMANN (12), RABAK (9), and WILLITS and KOKOSKI (11) have reported the quantitative reduction of the oil content in corn. McHARGUE (8) noted that the oil extracted from moldy cornmeal possessed a musty odor, was dark brown in color, and titrated 56.8 ml. alkali per kilogram of oil. Results similar to these were obtained for shelled corn by RABAK (9). A chemical analysis of the oil extracted from spoiled corn by that investigator revealed increases in specific gravity, refractive index, free acids, soluble acids, hydroxylated acids, and unsaponifiable constituents; decreases in volatile acids, insoluble acids and unsaturated acids were noted. WILLITS and KOKOSKI (11) observed higher saponification values and lower iodine numbers of the oil in spoiled cornmeal.

The relationship between the loss of organic matter and the formation of water in the molded corn indicated, as expected, that water was being liberated during mold development. This might suggest that molds would be able to create favorable moisture conditions for their own development even in initially fairly dry corn, providing that enough moisture was available in the kernels to allow them to initiate fungal development.

The changes in the fat acidity of molded corn reflected, in large measure, the acid-producing capabilities of the individual molds themselves. While the term "fat-acidity" implies that the origin of these fatty acid constituents resides in the degradation of the fats (7), its determination remains

quite empirical as it includes all the benzene-soluble acidic substances regardless of their origin. No distinction is made as to the extent to which these constituents arise directly from the fat of the corn itself and from the carbohydrates as by-products of mold metabolism. The recognized agents responsible for the formation of fatty acids from the fat have been (a) the enzymes normally present in the food product studied, (b) the direct participation of atmosphere oxygen, and (c) the fat-hydrolyzing lipases liberated by the microorganisms present. The present work suggests that perhaps part of the fat-solvent soluble acids produced in molding corn might include the acids produced by the microorganisms incident to their metabolism of corn carbohydrates.

. Summary

Pure cultures of 9 fungi isolated from naturally molded corn were grown in the laboratory for a 4-week period on steam-sterilized corn initially adjusted to approximately 32 percentage moisture content. Analyses were made at weekly and bi-weekly intervals for changes in the amounts of water, organic matter, fat acidity, pH, water-soluble, and -insoluble nitrogen. Seven of these fungi were also grown on Czapek-Dox liquid medium to compare their carbohydrate-utilizing and acid-producing powers on this medium with the changes they induced in corn.

Penicillium chrysogenum I, *P. chrysogenum* II, *Aspergillus niger*, and *A. flavus* were most active in decomposing corn organic matter, producing losses of 40% to 45% within a 4-week period. These same fungi on Czapek-Dox medium were initially the most rapid utilizers of glucose.

A positive linear relationship was found between the percentage loss in organic matter and the percentage of water (wet basis) in the moldy corn. A 1% decrease in organic matter resulted in a 0.55% increase of water (wet basis). The relationship between the quantity of organic matter lost and quantity of water formed proved linear over 2 and 4 weeks of fungus development. The ratios between these two variables were 1:0.701 at the 2-week period and 1:0.601 at the 4-week period.

Fat acidities in the corn were increased by all the fungi tested in this series of experiments. The greatest fat acidities were produced by the 4 fungi that decomposed organic matter the most rapidly, and by *Mucor racemosus* and *Aspergillus amstelodami* which decomposed the organic matter slowly.

Linear relationships were noted at the early period of fungus development between the losses of organic matter and the changes in the amounts of water-soluble and -insoluble nitrogen. Nonlinearity between organic matter loss and water-soluble nitrogen was noted at the later period.

General agreement between the pH and fat acidity of molded corn and the pH and titrable acidity of Czapek-Dox medium was obtained with the different fungi.

work and to Dr. W. G. Gaessler of the Plant Chemistry subsection for the determinations of fat acidities and nitrogen.

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CHANGES IN THE CHLOROPHYLL AND CAROTENE CONTENTS OF CURING BURLEY TOBACCO CUT AT DIFFERENT STAGES OF MATURITY[1]

R. N. Jeffrey and R. B. Griffith

Introduction

Changes in the chemical composition of burley tobacco which take place in curing are being investigated at the Kentucky Agricultural Experiment Station in an attempt to understand the physiology of curing and its effect on leaf quality. Color is recognized as one of the most important factors in the determination of leaf quality, any retention of green pigment in the cured leaf greatly reducing its value. The chlorophylls are the green pigments in normal tobacco, and the investigations reported in this paper were made primarily to determine the rate and amount of change in these pigments during air curing on the stalk of tobacco cut at three stages of maturity. The development of a method for determining carotene from the same solution used in the chlorophyll determinations made possible the study of this constituent.

This study of the catabolic changes of plant pigments is of general biochemical and physiological interest. Numerous articles have appeared reporting the rates of formation of these pigments under different conditions, but studies on pigment disappearance are rare. In 1918 Willstätter and Stoll (5) reported that the chlorophyll content of yellowed leaves was less than of green leaves. They did not, however, follow closely the loss of either chlorophyll or carotenoids. Guthrie (2) investigated the pigment changes in potted plants when placed in the dark as compared with others remaining in the light. His results indicated that tomato plants lost about 25% of their chlorophyll in four days, soybean plants about 70% in eight days, and yellow coleus practically none in eight days.

Methods

Harvesting and Curing Technique

Kentucky No. 16 burley tobacco was cut at three different stages of maturity as determined by experienced growers: (1) immature by about 10 days, (2) mature, and (3) overmature by 10 days. These lots of tobacco were topped to approximately 20 leaves, and to a height of about 136 cm. (4.5 ft.) two, 14, and 24 days respectively before cutting. The entire plant was cut just above the ground and the stalk "speared" onto a stick and hung to cure with the plant in an inverted position. Spearing

caused a split in the stalk about a foot long. Although the majority of the plant cells remained alive for some time, the more mature basal leaves, which had already started to turn yellow before cutting, died first, beginning at the tips. The yellowing and subsequent death of the cells then progressed gradually to the more immature leaves. The axillary buds near the tip of the stalk were still alive at the end of the curing period. Thus the curing process for this type of tobacco is one of gradual starvation and desiccation, accompanied by extensive chemical changes which may be catalyzed by the enzymes of the living plant.

The tobacco, after wilting in the field for 24 hours following cutting, was cured in the air-conditioned curing chambers described by O'BANNON (**4**) at 68% relative humidity and a temperature of 23.8° C. (74.9° F.). JEFFREY (**3**) found these conditions to be within the optimum range for the production of highest commercial quality cured leaf.

SAMPLING AND ANALYSIS

To sample two different stages of leaf maturity on each plant analytical samples were taken from two different levels. One, called the "basal leaf sample," was from each plant's basal three leaves which were sound and therefore likely to remain on the plant during curing; the other, designated as the "top leaf sample," was from the 5th, 6th, and 7th leaves from the point of topping. After curing, leaves from these positions on the stalk were graded chiefly as "trash" and "bright leaf," respectively. Leaves to be used as samples were measured and tagged in the field a day before cutting. Duplicate samples were taken in the field at cutting time, five days before the overmature plants were cut, and in the curing chambers. The frequency of sampling in the chambers was dependent on the rate of change observed. A sample consisted of four leaves, each taken from a different plant. The midribs were removed. The right halves of two of the leaves and the left halves of the other two were used for pigment extraction, and the remaining halves were placed in an oven at 65° C. for 48 hours to determine the percentage dry weight.

The leaf material was analyzed for chlorophylls *a* and *b* and for carotene using the method described by GRIFFITH and JEFFREY (**1**). By this method the pigments are extracted from the sample with acetone in a Waring blender and transferred to ether. Chlorophyll is determined in a dilution of the ether solution by means of spectrophotometric readings at the red absorption maxima of chlorophylls *a* and *b*, and carotene is determined spectrophotometrically after separation from the other pigments in the ether solution by the use of a chromatographic adsorption column. The estimation of xanthophyll had not been included in the method at the time this study was conducted.

EXPRESSION OF DATA

Since burley tobacco is cured on the stalk, the green or dry weight of the samples to be used for analysis could not be determined at cutting time.

YOUNG and JEFFREY (6) have shown that tobacco leaves cured under the same conditions used in this experiment lose 30% of their dry weight during the curing process; consequently, the oven dry weight does not constitute a satisfactory basis for reporting results.   They found, however, that there was a high degree of correlation between the product of length and width of leaf and leaf area, and that the leaf area was a satisfactory basis for reporting changes that occur in curing tobacco.   The leaf areas were calculated, using the equation :[2]

$$y = \frac{0.65\,x}{10,000} + 0.006$$

where y is the leaf area in square meters and x is the product of the length and width of the leaf expressed in centimeters.

Results

The chlorophyll and carotene contents of the top leaves at various times during the curing of plants cut when immature, mature and overmature are presented in table I.   Similar results for basal leaves are presented in table II.   The results expressed on an area basis are shown graphically

TABLE I

CHLOROPHYLL AND CAROTENE CONTENT OF TOP LEAVES AT VARIOUS STAGES OF CURING ON
THE STALK FOR TOBACCO PLANTS CUT AT THREE STAGES OF MATURITY.   RESULTS
CALCULATED ON THE BASIS OF OVEN DRY WEIGHT AND OF FRESH LEAF
AREA.   LEAVES IN CURING CHAMBER EXCEPT AS NOTED

AGE	DAYS FROM FIRST CUTTING	OVEN DRY WEIGHT	WEIGHT BASIS		CHLORO-PHYLL a	AREA BASIS	
			CHLORO-PHYLL	CARO-TENE		CHLORO-PHYLL	CARO-TENE
		%	mg./gm.	mg./gm.	%	mg./m.²	mg./m.²
	0.0*	16.2*	8.28 *	0.464*	72.0*	277.0*	15.4*
	2.0	16.1	7.18	0.444	70.1	227.0	14.0
	5.1	16.5	6.26	0.393	66.0	196.0	12.3
Immature ..	8.5	17.5	5.16	0.354	69.0	156.0	10.7
	15.1	24.6	1.46	0.263	68.5	39.0	7.0
	17.9	35.9	0.27	0.174	58.6	6.0	4.8
	21.0	51.4	0.52	0.183	67.3	13.0	4.5
	39.0	89.8	0.22	0.139	58.1	5.0	3.3
	10.0*	17.5*	5.10 *	0.299*	71.0*	188.0*	11.0*
	12.0	16.7	4.19	0.298	71.9	126.0	9.0
	15.1	17.8	2.45	0.190	64.3	78.0	6.1
Mature	18.5	34.4	0.24	0.119	67.1	6.4	3.2
	28.3	86.7	0.022	0.075	62.2	0.6	2.0
	34.0	86.1	0.020	0.070	58.3	0.5	2.0
	10.0†	17.5†	5.10 †	0.299†	71.0†	188.0†	11.0†
	14.8†	15.6†	4.46 †	0.265†	72.6†	160.0†	9.5†
	20.0*	16.6*	2.76 *	0.200*	72.6*	92.0*	6.7*
Overmature ...	21.9	18.5	1.88	0.156	71.6	62.0	5.2
	24.5	30.2	0.21	0.089	64.2	6.3	2.7
	27.9	79.1	0.027	0.079	61.4	0.8	2.3
	39.0	89.3	0.014	0.064	57.4	0.4	1.9

* Cut.
† In field.

[2] An error was present in the previously published form of this equation (6).

in figure 1. The left part of this figure shows the changes which take place in the chlorophyll content of the top leaves. In the first 17.9 days after cutting, the chlorophyll content of the immature tobacco decreased at an average rate of 15.1 mg./m.² per day. Chlorophyll was lost by the mature tobacco at the rate of 21.4 mg./m.² per day in the first 8.5 days of curing, and by the overmature tobacco at the rate of 16.9 mg./m.² per day in the first 5.1 days of curing. The rate of chlorophyll loss in the tobacco growing in the field was 8.9 mg./m.² per day in the first 10 days and 9.6 mg./m.² per day in the second 10 days. The tobacco cut when immature and that
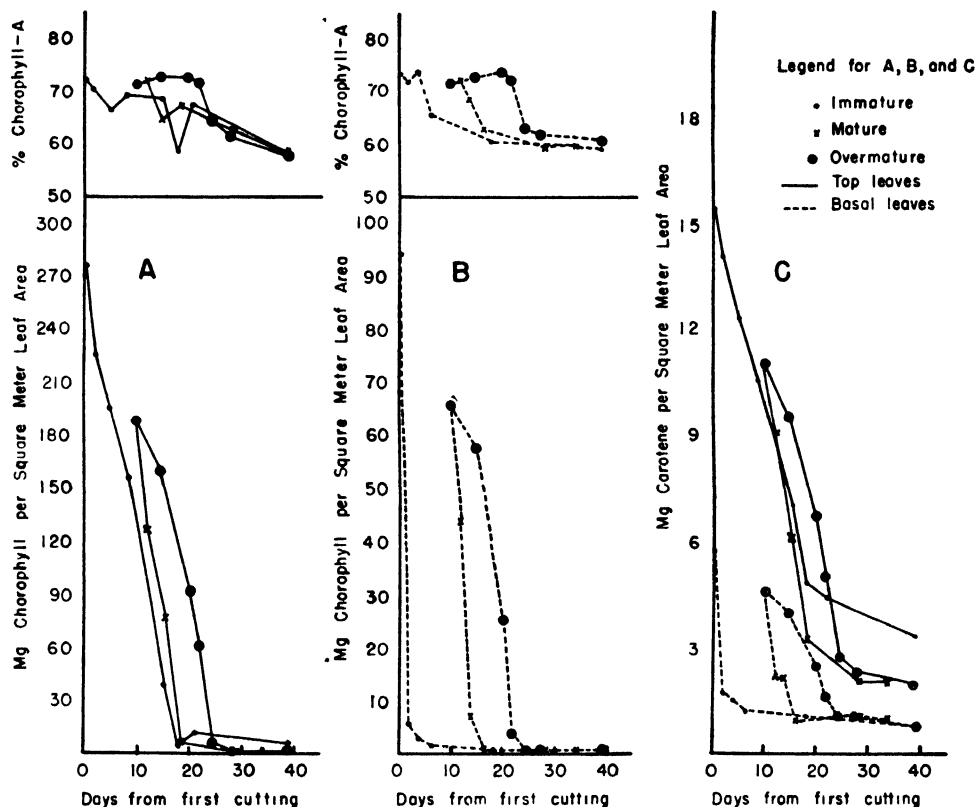
FIG. 1.   Chlorophyll changes in top leaves (A), in basal leaves (B), and carotene changes in top and basal leaves (C) of curing burley tobacco cut when classified by experienced growers as immature, mature (10 days later), and overmature (20 days later).

cut when mature reached essentially the same chlorophyll content at the same time, even though the mature tobacco was cut 10 days later. Less time was required for chlorophyll destruction in the mature and overmature tobacco than in the immature tobacco, curing was more uniform, and the quality of leaf was better. The top leaves of the tobacco cut while immature still contained chlorophyll at the end of the curing period, incipient house-burn had occurred, and even the quality of the basal leaves was low.

The changes in the percentage of chlorophyll *a* based on total chlorophyll, are shown in the upper part of figure 1. The point representing the immature tobacco after 21 days is probably in error, since in the other five

instances in this and in the next graph representing basal leaves, the percentage chlorophyll *a* fell from about 70% to below 63% at about the same time that the total chlorophyll concentration ceased to fall. The oven dry weight in the leaf web was about 30% to 35% in all three cuttings of tobacco when the total chlorophyll content and the percentage chlorophyll *a* approached minimum values.

Changes in the chlorophyll content of the basal leaves are presented in the central part of figure 1. The initial chlorophyll content of these older leaves was much lower than that of the top leaves, and the time required

TABLE II

CHLOROPHYLL AND CAROTENE CONTENT OF BASAL LEAVES AT VARIOUS STAGES OF CURING ON THE STALK FOR TOBACCO PLANTS CUT AT THREE STAGES OF MATURITY. RESULTS CALCULATED ON THE BASIS OF OVEN DRY WEIGHT AND OF FRESH LEAF AREA. LEAVES IN CURING CHAMBER EXCEPT AS NOTED

AGE	DAYS FROM FIRST CUTTING	OVEN DRY WEIGHT	WEIGHT BASIS		CHLORO-PHYLL *a*	AREA BASIS	
			CHLORO-PHYLL	CARO-TENE		CHLORO-PHYLL	CARO-TENE
		%	mg./gm.	mg./gm.	%	mg./m.²	mg./m.²
Immature	0.0*	11.3*	3.29*	0.211*	73.0*	94.2*	6.05*
	2.0	13.5	0.23	0.069	71.7	5.9	1.73
	3.9	31.0	0.11	0.051	73.1	3.3	1.57
	6.4	47.1	0.06	0.042	65.4	1.5	1.20
	18.1	86.9	0.03	0.043	60.1	0.8	1.02
	39.0	89.8	0.03	0.030	59.0	0.7	0.73
Mature	10.0*	12.3*	2.26*	0.158*	71.2*	65.5*	4.57*
	12.0	13.7	1.77	0.087	71.3	43.6	2.16
	13.9	21.2	0.30	0.085	67.9	7.4	2.07
	16.4	79.8	0.06	0.042	62.8	1.3	0.91
	28.3	87.4	0.03	0.043	59.0	0.8	1.06
	34.0	88.0	0.03	0.043	59.9	0.7	0.96
Overmature	10.0†	12.3†	2.26†	0.158†	71.2†	65.5†	4.57†
	14.8†	11.8†	1.71†	0.121†	72.5†	57.5†	4.02†
	20.0*	11.9*	0.92*	0.090*	73.6*	25.4*	2.47*
	21.9	20.7	0.16	0.063	72.0	4.0	1.58
	24.5	79.9	0.04	0.041	62.9	0.8	1.00
	27.9	86.0	0.04	0.043	61.5	1.0	1.06
	39.0	90.5	0.02	0.027	60.5	0.4	0.67

* Cut.
† In field.

for its loss was less than in the top leaves. In the first two days after cutting, the rate of chlorophyll loss in the basal leaves of the plants cut while immature was 44.3 mg./m.² per day. In the first 3.9 days of curing the rate of loss in the basal leaves of the mature tobacco was 14.9 mg./m.² per day. While the overmature tobacco was still in the field the rate of loss was 4.0 mg./m.² per day and in the first 1.9 days after cutting the rate of loss was 11.3 mg./m.² per day. Except for the immature tobacco the rate of loss per day in the basal leaves was less than the rate of loss in the top leaves, but because of lower initial chlorophyll values the length of time necessary for chlorophyll decomposition was less. The extremely rapid rate of 44.3 mg./m.² per day of chlorophyll loss in the immature basal leaves was over

twice as great as any other rate of loss.   Translocation of some substance or substances to the immature top leaves is the only explanation that can be offered for this rapid rate.   The smaller rate of loss in the basal leaves of the plants cut when mature or overmature was probably due to the greater maturity and lower initial chlorophyll content of these plants.   After five days of curing in plants of all stages of maturity there was very little change in chlorophyll content of the basal leaves.

The changes in percentage of total chlorophyll of the basal leaves which is chlorophyll *a* are shown in the upper part of figure 1.   The sharp drop in percentage chlorophyll *a* in these basal leaves corresponded much more definitely than in the top leaves to the time at which the total chlorophyll reached a low value.   It may be seen that the dry matter at this time is about 30% to 35% (table II), as it was also in the top leaf samples.   However, decrease in chlorophyll values does not cease altogether at this point.   In these experiments the chlorophyll concentrations reached a low value some time before the leaves became dry.   Other experiments in the curing chambers have indicated that this would not have been true if the relative humidity of the air surrounding the curing plants had been low instead of optimum.

The results of the carotene determinations are presented in figure 1, C. The upper group of three curves represents the changes in the top leaves, while the lower group of three curves represents the changes in the basal leaves.   Each set of curves is similar to the corresponding set of chlorophyll curves; however, the carotene does not appear to be so rapidly nor so completely destroyed as is the chlorophyll.   Thus the yellowing of tobacco in the field and in curing is due in part to a more rapid and complete disappearance of chlorophyll than of carotene.   At the time the break in the chlorophyll concentration curve occurs, the carotene content has been reduced to about one quarter its former value.   A break occurs in the carotene concentration curves at this same time, corresponding to 30% to 35% dry matter, but these curves show a greater downward trend after this point has been reached than do the chlorophyll curves.

Discussion

The decrease in the calculated chlorophyll *a* percentage shown in the upper part of figure 1 may not appear to be very large, but there is reason to believe that the actual decrease is greater than is shown by these figures. All chlorophyll concentrations and percentage chlorophyll *a* values were calculated by means of equations based on the spectrophotometric absorption values at the red maxima of pure chlorophylls *a* and *b*.   In green tobacco leaves no pheophytins were found, so satisfactory values were obtained by means of these equations.   A chromatographic study of the pigments present in cured tobacco showed evidence of more pheophytin *a* than of chlorophyll *a*, though no pheophytin *b* was found.   The absorption of, pheophytin *a* at the red maximum of chlorophyll *a* is about half the latter, which would result in a calculated chlorophyll concentration by the method used, larger

than the true concentration of chlorophyll *a* plus *b*, but smaller than the concentration of the chlorophyll plus pheophytin *a*. This was confirmed by the concentration values calculated from the absorption values at 600 mμ, where the absorption of pheophytin *a* was more nearly equal to that of the chlorophylls. Close agreement was obtained between total chlorophyll concentration calculated from the readings at 600 mμ and at the *a* and *b* maxima on fresh leaf, but on the cured tobacco samples the concentration values determined at 600 mμ were larger than those calculated by the equations based on the chlorophyll maxima. Similarly, the percentage of chlorophyll *a* calculated by means of the equation is larger than the percentage of the chlorophyll which is really chlorophyll *a;* consequently the change in percentage chlorophyll *a* is really greater than that shown in the tables.

The break in the carotene curves is just as definite as that in the chlorophyll curves but the cause is more difficult to explain. At the time the break occurred the carotene values were about one-fourth as high as in the corresponding samples at cutting time. It does not seem probable that this concentration was low enough to affect the rate of the reaction sharply. Nor does it seem probable that the moisture content was low enough to cause such a marked reduction in reaction rate. The oven dry weight at this time was 30% to 35% and moisture was 65% to 70%. The moisture content threshold below which many other chemical reactions in cured tobacco leaf are inhibited, is approximately 80% dry matter and 20% moisture. It is possible that the carotene had undergone some change, but it was still sufficiently similar to true *beta* carotene to behave normally in the magnesium oxide-celite adsorption column. No light absorption studies were made of the carotene fraction from cured tobacco. Another possible explanation of the results is that the rate of carotene destruction may be connected in some way with the chlorophyll concentration, since the break in the two curves seems to occur at the same time. This could be a case of catalysis or it could be related to the existence of a definite chromoprotein molecule containing both chlorophyll and carotene, such as has been suggested by many workers.

Summary

1. A study was made of the changes in chlorophyll and carotene content of the leaves of stalk-cut burley tobacco during the air-curing process. Leaves from the upper and lower parts of plants, cut when immature, mature, and overmature, were analyzed. The initial chlorophyll content of the leaves decreased with increasing maturity, and was higher in the upper than in the corresponding basal leaves.

2. The rate of loss of chlorophyll in the top leaves was not greatly affected by maturity, and as a result, low chlorophyll concentrations were reached in a shorter time of curing in the overmature than in the immature plants.

3. The highest rates of chlorophyll loss were observed in the basal leaves of immature plants, indicating that translocation of some substances to the immature leaves may have occurred.

4. As the total chlorophyll content of the leaves reached low values, the percentage of chlorophyll *a* based on total chlorophyll decreased.

5. The shapes of the curves showing the carotene content of the leaves were very similar to the corresponding chlorophyll curves for leaves of each degree of maturity, though the carotene did not disappear so completely as the chlorophyll.

6. A change in rate of loss of carotene and of chlorophyll and in the proportion of chlorophyll *a* all occurred at a fairly definite moisture content, though the time required to reach this point depended upon the maturity of the leaves.

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INFLUENCE OF TEMPERATURE ON GROWTH AND ALKALOID CONTENT OF CINCHONA SEEDLINGS

HAROLD F. WINTERS, ARNAUD J. LOUSTALOT, AND NORMAN F. CHILDERS

(WITH FOUR FIGURES)

Introduction

The importance of temperature as an environmental factor in the growth of Cinchona, the tree from which quinine is obtained, may be realized from a study of its natural environment. The genus is native to a region extending over 1,500 miles along the slopes of the Andes Mountains of South America. The various species occur at altitudes ranging from 2,500 to 9,000 feet above sea level (4). Relatively cool temperatures, high rainfall and high humidity prevail in these areas. In Java, where Cinchona has reached the peak of cultivation, the most suitable elevation is from 3,000 to 7,000 feet (1). Temperature conditions are relatively uniform from one month to the next. A mean temperature of 63° F. was the average over a period of 11 years at the Government Cinchona Station located at Tjinjiroean, Java, at 5,200 feet elevation (2).

Materials and methods

Three air-conditioned chambers 7 by 9 and 7 feet high, were constructed within a standard even-span greenhouse (fig. 1). The chambers were built

FIG. 1. (Left) Three air conditioned chambers in which Cinchona seedlings were grown. (Right) Interior of one chamber showing temperature-control equipment and seedlings.

so that one-half their height was below the ground floor of the greenhouse. The tops and part of the sides of the chambers consisted of double glass with partitioned dead air spaces between.

The desired temperatures were uniformly maintained within each chamber by standard air-conditioning equipment. Fans on the diffusers operated continuously. When a temperature higher than the prevailing outside temperatures was desired in a chamber, small heating coils were turned on and the refrigeration system operated against this small flow of heat, thus main-

TABLE I

APPEARANCE OF *Cinchona ledgeriana* AND *C. pubescens* SEEDLINGS GROWN AT THREE TEMPERATURE LEVELS

EN-VIRON-MENT	TEMPERATURE				*C. ledgeriana*	*C. pubescens*
	SUMMER*		WINTER†			
	NIGHT	DAY	NIGHT	DAY		
	°F.	°F.	°F.	°F.		
Warm chamber	70	80	65	80	Many necrotic spots on older leaves during summer. No necrotic spots at end of experiment. Older leaves shed in summer. Thin leaves. Stems slender and weak. Poor root development.	Vigorous strong stems. Large leaves, tending to shade the weaker plants. Long internodes. Good root development.
Medium chamber	65	75	60	75	Occasional necrotic spots during summer, none in winter. Vigorous, strong growth. Good root development.	Sturdy healthy plants. Good root development.
Cold chamber	60	70	55	70	Stocky plants with thick stems and undulate leathery leaves. Leaves tinged with red. Excellent root development.	Stocky plants. All leaves retained. Short internodes. Excellent root development.
Green-house‡					Many necrotic areas on older leaves in summer. Plants shed all but 1 or 2 pairs of leaves. Poor root development.	

* Maintained from April through September.
† Maintained from October through March.
‡ No control.

taining a straight-line temperature level. During the first few months of the experiment it was necessary to use the coils for about three hours each morning in order to gradually raise air temperatures in the chambers to the desired summer-period levels.

Temperatures were thermostatically regulated for night and day and for summer and winter (table I). Thermostat settings were made manually at 8:00 A.M. and 4:00 P.M. daily. About 6 hours were usually required for the temperature in the chambers to reach the night levels, due to the simultane-

ous heavy drain on the compressor by all chambers. The day temperatures were reached in 2 to 4 hours depending upon the sky conditions.

Several times during the experiment leaks developed and the system was out of operation for short periods, during which time all plants were
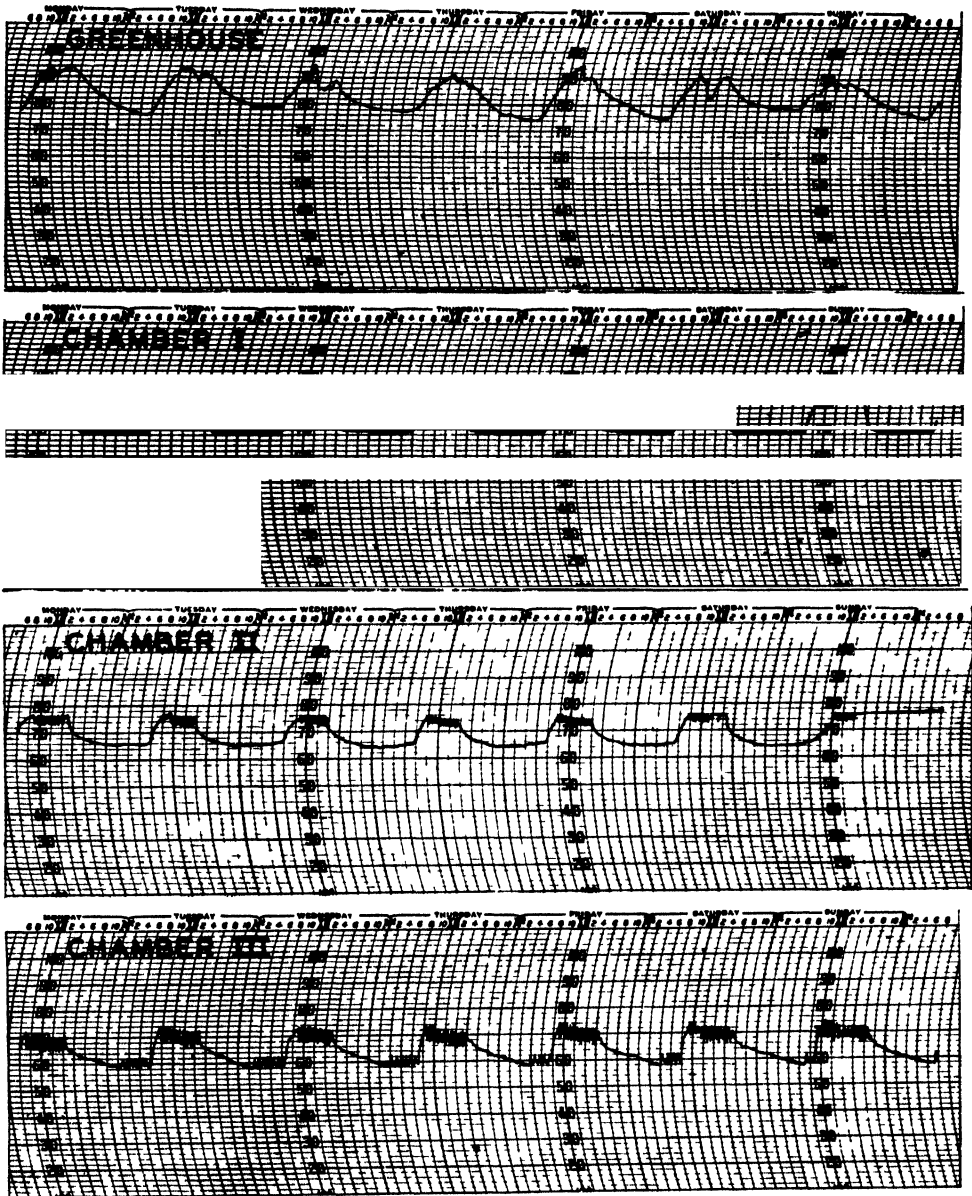
FIG. 2. A typical chart of temperatures in the greenhouse outside the chambers and sample temperature charts from warm, medium, and cold chambers. Data taken during summer period; in winter period night temperatures were 5° lower in chambers.

subjected to outside air-temperature conditions. As a whole, however, the experimental temperatures were maintained fairly closely. Continuous thermographic records were kept in each chamber and representative charts from the chambers and the outside greenhouse are given (fig. 2). Relative

humidity was maintained as near 100% as possible by spraying the floors with water.

Concrete benches within the chambers were filled with a soil composed of equal parts of loamy-clay soil, silica sand, peat moss, and leaf mold. Forty seedlings of *Cinchona ledgeriana* Moens and 40 of *C. pubescens* Vahl were planted in each chamber in March, 1945. Forty seedlings of *C. ledgeri-*

FIG. 3. *C. ledgeriana* seedlings grown under different temperature conditions: (1) warm chamber (70° to 80° F.), (2) medium chamber (65° to 75° F.), (3) cold chamber (60° to 70° F.), and (4) greenhouse bench where air temperature was usually on a higher level than that of the warm chamber. Note well developed root system on typical plant from cold chamber (3) and sparse root system on plant from warm chamber (1). Stems and foliage of seedlings grown in the cold chamber were rigid; the tips and lower sides of the leaves had a reddish color.

ana were also placed in the greenhouse outside the chambers for comparison with the plants of the same species grown under controlled conditions. These were once-transplanted seedlings of uniform 4-inch height grown in a sheltered nursery at Maricao, Puerto Rico. Spacing in the greenhouse beds was 6 by 6 inches. Plants that died during the first weeks of the experiment

were replaced immediately in order to provide as nearly as possible a uniform stand.

On several occasions thrips and aphids appeared on the plants. Both were easily controlled by applications of derris dust containing 1% to 2% rotenone. Eradication of aphids was more difficult in the cold chamber than in the warm or medium temperature chamber.

Final observations and growth data were taken on February 25, 1946. The plants were removed from the beds and all excess soil washed from root systems. Total fresh and oven dry weights were recorded for each plant and separately for leaves, stems, and roots. Chemical analyses were made for total alkaloids and quinine in the stems and roots, by the method described by LOUSTALOT and PAGÁN (3).

Results

The two species of *Cinchona* responded quite differently to the four environments (table I and figs. 3 and 4).

FIG. 4. Representative seedlings of *C. pubescens* grown under different temperature conditions. Left to right: warm chamber (70° to 80° F), medium chamber (65° to 75° F.), and cold chamber (60° to 70° F.).

C. LEDGERIANA

In the warm chamber *C. ledgeriana* produced weak, slender stems and poor root systems. During the summer period many necrotic spots developed on the leaves. A more vigorous healthy growth was produced in the medium temperature chamber In the cold chamber a stocky growth developed, characterized by thick stems, vigorous root systems, and undulate red tinged leaves. Growth was poorest in the greenhouse outside the chambers where temperatures averaged 5 to 10 degrees higher than in the warmest chamber. During the summer, large necrotic areas developed on the leaves

TABLE II

GROWTH DATA OF *C. ledgeriana* AND *C. pubescens* GROWN AT THREE
TEMPERATURE LEVELS

ENVIRON-MENT	C. ledgeriana					C. pubescens				
	FRESH WEIGHT*	DRY WEIGHT*	DRY MATTER†	HEIGHT*	SURVIVAL	FRESH WEIGHT*	DRY WEIGHT*	DRY MATTER†	HEIGHT*	SURVIVAL
	gm.	gm.	%	cm.	%	gm.	gm.	%	cm.	%
Warm	26.23	5.48	20.89	54.2	86.36	99.92	18.73	18.75	74.6	56.81
Medium	40.27	8.36	20.76	70.6	90.00	79.09	13.26	16.76	67.5	82.50
Cold	34.39	7.13	20.73	46.7	85.00	60.75	9.05	14.90	45.2	100.00

* Average per plant.
† Concentration of.

and subsequent defoliation left the plants almost leafless. Root development under these conditions was extremely poor (fig. 3).

Cinchona ledgeriana seedlings grown in the medium temperature chamber made the best growth as indicated by the average height and average fresh and dry weights per plant (table II). Seedlings grown in the warm chamber were taller, on the average, than those in the cold chamber, but they had an average fresh and dry weight less than the cold chamber plants. Plants grown in the greenhouse outside the chambers were inferior in growth to all plants grown in the chambers. These differences were all statistically significant at the 5% level and in some cases at the 1% level. There was no appreciable effect of temperature on the concentration of dry matter or on the percentage of plants surviving in the three chambers.

There was a definite effect of temperature on the shoot-root ratio; seedlings from the warm chamber had the highest ratio and those from the cold chamber the lowest (table III). The table also shows that the proportion of leaves by dry weight to the rest of the plant was not appreciably different in any of the treatments. Plants grown in the cold chamber, however, had

TABLE III

EFFECT OF TEMPERATURE ON PROPORTIONAL DRY WEIGHT OF LEAVES, STEMS, AND
ROOTS OF *C. ledgeriana* AND *C. pubescens* SEEDLINGS GROWN
AT THREE TEMPERATURE LEVELS

ENVIRON-MENT	C. ledgeriana					C. pubescens				
	LEAVES	STEMS	ROOTS	SHOOT-ROOT RATIO	BARK ON STEMS	LEAVES	STEMS	ROOTS	SHOOT-ROOT RATIO	BARK ON STEMS
	%	%	%	%	%	%	%	%	%	%
Warm	43.61	43.97	12.42	7.01	20.44	33.74	48.85	17.41	4.75	20.84
Medium	41.38	45.45	13.17	6.58	23.10	41.18	43.14	15.68	5.39	20.66
Cold	42.91	40.67	16.42	5.10	29.00	42.88	35.24	21.88	3.56	23.06

a lower percentage of stem but higher percentage of bark than plants grown in either of the other chambers.

Under the conditions of this experiment there was little or no effect of temperature on the percentage of total alkaloids and quinine in the stems and roots of young *C. ledgeriana* seedlings (table IV). Figure 3 shows a

TABLE IV

TOTAL ALKALOID AND QUININE CONTENT IN ROOTS AND STEMS OF *C. ledgeriana* AND *C. pubescens* SEEDLINGS GROWN AT THREE DIFFERENT TEMPERATURES

ENVIRON-MENT	C. ledgeriana				C. pubescens			
	TOTAL ALKALOIDS		QUININE SULPHATE		TOTAL ALKALOIDS		QUININE SULPHATE	
	STEMS	ROOTS	STEMS	ROOTS	STEMS	ROOTS	STEMS	ROOTS
	%	%	%	%	%	%	%	%
Warm	2.79	5.20	0.80	1.80	3.12	5.24	1.0	1.45
Medium ...	2.69	3.38	0.50	2.10	2.97	4.45	0.7	1.30
Cold	2.92	4.21	0.93	1.90	2.60	3.10	0.9	0.70

representative *C. ledgeriana* plant from each chamber and also a seedling grown in the greenhouse bench where it was subjected to the natural fluctuations of greenhouse temperature.

C. PUBESCENS

The *C. pubescens* species of Cinchona is morphologically different from *C. ledgeriana* (table I and figs. 3 and 4). There was a direct correlation between growth and temperature (table II). The best and most vigorous growth as shown by height and weight measurements was produced in the warm chamber. The cold chamber produced short stocky plants with short internodes and excellent root systems. Plants from the medium temperature chamber were intermediate in size and vigor compared to those from the warm and cold chambers.

Percentage of survival for *C. pubescens* was inversely correlated with temperature, and all differences were of a high order of significance. In the warmer chambers competition due to the large leaves and close spacing of the plants apparently resulted in death of the weaker plants, while in the cold chamber where growth was slower and competition less, 100% survived. This is a contrast to the uniform survival in all chambers of the narrow-leaved *C. ledgeriana* where competition for light evidently was not a factor.

Unlike *C. ledgeriana* the concentration of dry matter in *C. pubescens* seedlings was directly correlated with temperature. The plants in the warm chamber produced the highest percentage of dry matter and those in the cold chamber the lowest. There was no significant temperature effect on shoot–root ratio (table III). The proportion of leaves to total plant on a dry weight basis was lowest in the warm chamber and highest in the cold

chamber, whereas the reverse was true with percentage of stems. As with *C. ledgeriana,* the percentage of bark on the stems was somewhat higher on seedlings grown in the cold chamber than on those grown in the warmer chambers.

The percentage of total alkaloids in stems and roots and quinine in the roots was directly correlated with temperature (table IV). The percentage quinine in the stems of plants grown in the medium temperature chamber was somewhat lower than that of plants grown in the warm and cold chambers.

Discussion

The results obtained in the experiment emphasize the importance of temperature as a factor influencing the growth and composition of Cinchona. A comparison of the responses of the two species to relatively small differences in temperature (5° to 10° F.) suggests that *C. pubescens* is better adapted to warmer temperatures than is *C. ledgeriana* which grows best at cool temperatures and is more exacting in its requirements. Of the two species, the *C. pubescens* was much more vigorous irrespective of temperature conditions. This may be due to its inherent ability to produce vigorous root systems under varied conditions. Development of the root systems of *C. ledgeriana* was adversely affected by higher temperatures.

Although plants of *C. ledgeriana* grown in the warm chamber made more linear growth than those from the cold chamber, the plants in the cold chamber had a greater average dry weight. The beneficial effect of the cold temperature in producing a higher percentage of bark for both species is offset by the smaller total dry weight produced under cool conditions.

Summary

1. *Cinchona ledgeriana* and *C. pubescens* were grown under three temperature controlled conditions ranging between 70° to 80° (warm), 65° to 75° (medium), and 60° to 70° F. (cold). In order to simulate natural conditions in each chamber, the night temperature was lowered 5° from October through March.

2. There was a marked difference in response of the two species to temperature. The *C. pubescens* seedlings grown in the warm environment had an average dry weight about twice that of plants grown in the cold treatment, while plants grown in the medium temperature environment were intermediate. There was a similar trend in height of plants.

The *C. ledgeriana* seedlings grew best in the medium temperature and were more exacting in their temperature requirements than *C. pubescens.*

3. There was no appreciable effect of temperature on percentage survival of *C. ledgeriana* seedlings whereas the percentage survival of *C. pubescens* was lower at the higher temperature level.

4. The shoot–root ratio of the *C. pubescens* plants was not significantly affected by temperature. The *C. ledgeriana* seedlings, however, were affected

by temperature; the lower the temperature the greater the roots in proportion to the shoots.

5. High total alkaloids and quinine in *C. pubescens* roots and stems seemed to be associated with high temperature and vigorous growth. There was no consistent trend in the *C. ledgeriana* seedlings in regard to these constituents.

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A COMPARISON OF PHOTOSYNTHESIS IN INDIVIDUAL PINE NEEDLES AND ENTIRE SEEDLINGS AT VARIOUS LIGHT INTENSITIES

PAUL J. KRAMER AND WALTER S. CLARK

(WITH THREE FIGURES)

It was found by KRAMER and DECKER (5) that loblolly pine seedlings have a relatively lower rate of photosynthesis at low light intensities than do certain species of competing hardwoods. Seedlings of dogwood, eastern red oak, and white oak attained a maximum rate of photosynthesis at an intensity equal to about one-fourth of full sunlight (2500 foot candles) and maintained an approximately constant rate at all higher intensities. Loblolly pine seedlings attained only about two-thirds their observed maximum rate at one-fourth of full sunlight, and the rate continued to increase to the highest intensity used, which was 9300 fc.

Various reasons might exist for the difference in behavior. There might be a fundamental difference in the photosynthetic mechanism of the pines and hardwoods, or the stomates of pine might require more light to open fully than do the stomates of hardwoods. Either situation could at least partly account for the observed difference. Consideration of certain published works suggested that mutual shading among the pine needles might account for the observed difference. HEINICKE and CHILDERS (3) found that individual, fully exposed leaves of apple attained their maximum rate of photosynthesis at one-fourth to one-third of full sun, but photosynthesis of entire trees was limited by light intensity up to full sunlight. This, they stated, indicated that a large part of the leaf area of an apple tree is too heavily shaded to carry on maximum photosynthesis except during the middle of very bright days. UHL (7) reported that in comparing the rates of photosynthesis of two-, three-, and five-needled species of pines the rate of photosynthesis per unit of photosynthetic tissue decreased as the number of needles per fascicle increased. Thus the rate of photosynthesis of two-needled pines was greater than that of five-needled pines, because the needles of the latter shade each other more. It was decided, therefore, to find whether the shading of some needles by other needles might be one cause of the relatively low rate of photosynthesis of loblolly pine seedlings exposed to light of low intensity.

Methods

The plants used were two-year-old loblolly pine (*Pinus taeda* L.) seedlings grown in pots out-of-doors in full sun. Fully matured needles of the current year's growth were used in all experiments to avoid variations in behavior caused by differences in age of needles. Mutual shading of the needles was prevented by arranging them in small leaf chambers (fig. 1).

51

The chambers were constructed of cellulose acetate sheet in such a manner that the needles of four fascicles could be inserted through the holes at each end and held perpendicularly to a light source and far enough apart that they could not shade each other. Details of their construction are shown (fig. 2). Each leaf chamber held a four-cm. segment of each of 12 needles, having a total leaf surface of about 2 3 cm , and three leaf chambers were attached to each seedling The air temperature within each leaf chamber
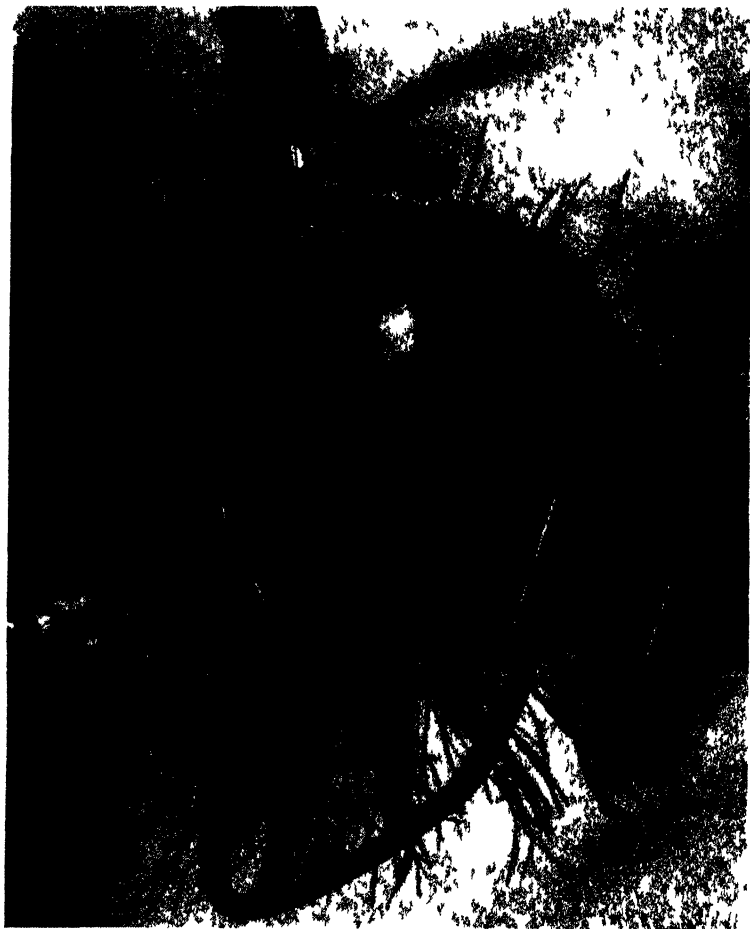
FIG. 1 View from above showing leaf chambers in place on loblolly pine seedling

was maintained at 25° ± 1 5° C. by pumping cold water through the water jacket on the lower side of the chamber. Thermocouples were inserted in the chambers to measure the air temperature. The potted seedlings with their attached leaf chambers were placed in the constant temperature chamber described by DECKER (2) and kept at 26° ± 2° C. The light source was a battery of projector-type Mazda lamps which provided a maximum intensity of about 9300 fc below the top of the chamber. The leaf chambers were oriented with respect to the light source so they were always in the same position and at the same distance from it. The seven light intensities used were obtained by means of various combinations of shades made from

screen wire and cheesecloth.   Each seedling was exposed to all seven intensities in one day, the sequence being randomized for each seedling to eliminate any systematic effect of sequence of intensities on the results.   The period of exposure to a given light intensity was one hour, and a fifteen-minute adjustment period was allowed following each change in intensity.   Fourteen plants were used, giving fourteen independent observations at each light intensity.

Apparent photosynthesis was measured by determining the difference in carbon dioxide content of an air stream before and after it had passed through the leaf chamber.   The air streams from the three leaf chambers on a single plant were combined and passed through a spiral absorption tower
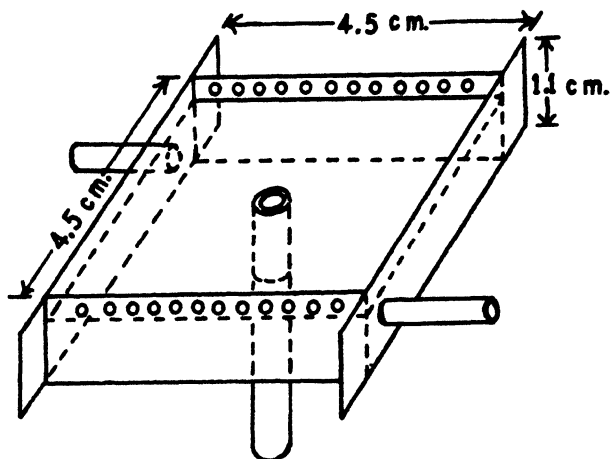
Fig. 2.   Drawing of leaf chamber with cover removed.   It is constructed of cellulose acetate sheet about 0.7 mm. thick, the pieces being cemented together with Duco cement. Cold water is circulated through the lower part of the chamber by means of the tubes projecting through the sides.   Air enters through the same holes in the ends of the upper section which hold the needles, and it passes out through the tube in the center.   Pieces of rubber band (not shown) were cemented to the upper edges of the leaf chamber to serve as a gasket for the cover which is a rectangular piece of cellulose acetate held in place by rubber bands slipped over the strips projecting at each end.

designed by the junior author as a modification of one described by LEACH, MOIR and BATHO (6).   The tower contained 50 ml. of 0.01 N NaOH, and the change in concentration of the alkali caused by absorption of carbon dioxide was measured by means of the change in its electrical resistance.   With the concentration of alkali, time interval and rate of air flow used in these experiments about 0.1 mg. of carbon dioxide could be measured.   This represented about 2% of the total carbon dioxide absorbed by a tower.   The rate of air flow was adjusted so that not over 15% of the carbon dioxide was removed by the pine needles, because it has been found that the rate of photosynthesis decreases rapidly when more than this is removed (2).

The volume of air passed over the pine needles was measured by means of calibrated flowmeters placed between the leaf chambers and the absorption towers.   A simple automatic pressure regulating valve was devised to

maintain constant pressure on the vacuum line (1). Three absorption towers were operated simultaneously, two being used to measure the carbon dioxide content of air streams from two plants and the third to measure the carbon dioxide content of the air before it passed over the needles.

Results

The results of the experiments are summarized in table I and fig. 3. The rate per unit of leaf surface was calculated on the assumption that all samples of leaves had the same area, 2.3 sq. cm. If any error resulted from this assumption, it did not enter into the comparisons between light intensities because all such comparisons for each plant were made on the same leaf tissue. It is possible to compare the curve for apparent photosynthesis of individual pine needles with those for entire pine seedlings and entire hard-

TABLE I

RATES OF APPARENT PHOTOSYNTHESIS OF LOBLOLLY PINE NEEDLES AT VARIOUS LIGHT INTENSITIES. EACH VALUE IS THE AVERAGE OF 14 INDEPENDENT OBSERVATIONS ON 14 PLANTS

LIGHT INTENSITY	CO_2 ABSORBED PER 6.9 CM.^{2} PER HR.	RATE OF OBSERVED MAXIMUM
fc	*mg.*	*%*
375	0.277*	26.7
2250	0.721	69.6
3550	1.036	100.0
4350	0.934	90.2
6000	0.876	84.6
7550	0.882	85.1
9200	0.887	85.6

* The standard error of the difference between any two of these values is 0.103 and differences of twice this value are significant at the 5% level.

wood seedlings (fig. 3). There is an obvious difference between the behavior of entire pine seedlings and that of individual pine needles. The rate of photosynthesis of entire pine seedlings increased with increasing light intensity up to the brightest light used, but the rate of photosynthesis of individual, fully exposed pine needles increased only up to about one-third of the highest intensity used and then remained unchanged with further increase in light intensity. In this respect their behavior is similar to that of hardwood seedlings which are represented by the curve for eastern red oak (fig. 3). The data for entire pine and oak seedlings are from the work of KRAMER and DECKER (5).

Statistical analysis of the data for pine needles showed the increase in rate of photosynthesis from 330 to 3350 fc to be highly significant, but no significant difference exists between the rates at 3350 and 9200 fc. The rate of photosynthesis of entire pine seedlings was significantly higher at 9300 fc than at 3300 fc. There seems to be, therefore, a real difference between the behavior of the entire pine seedlings and that of the individual needles.

It seems possible that this difference in behavior can be attributed largely

to the greater amount of mutual shading which occurs among pine needles than among the leaves of most hardwood seedlings.   No satisfactory method has been devised to measure mutual shading, but inspection of the leaf arrangement of dogwood and oak seedlings indicates that since the leaves form an umbrella-shaped mosaic they shade each other very little when illuminated from above.   Pine needles, on the other hand, are arranged radially along several inches of stem and, therefore, shade each other.   Pre-
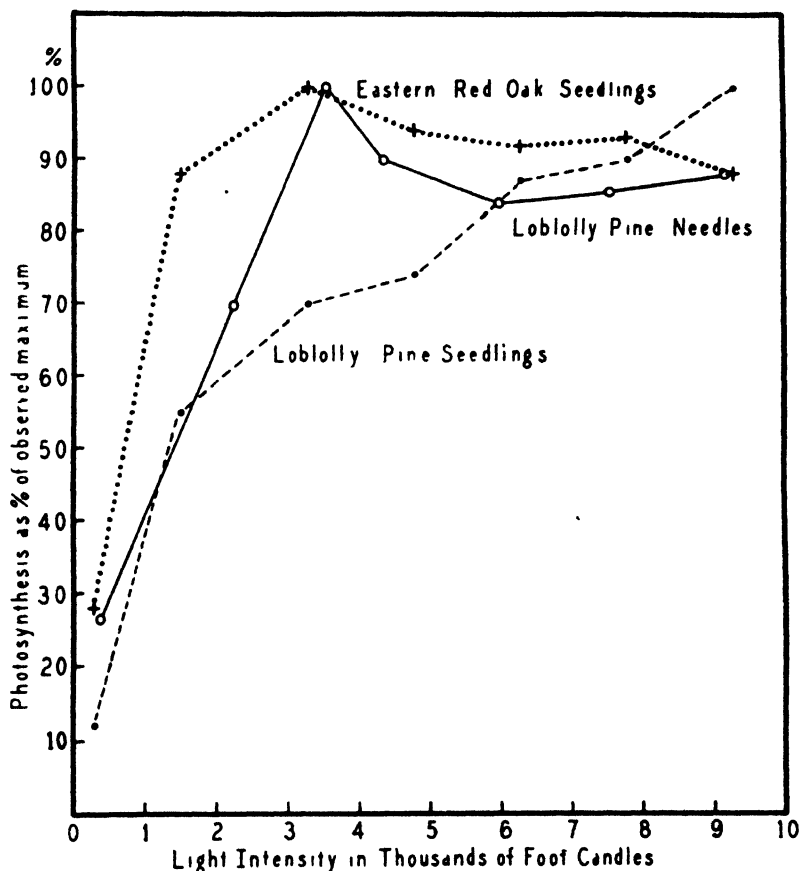
FIG. 3.   Rates of photosynthesis of individual loblolly pine needles, entire loblolly pine seedlings, and eastern red oak seedlings at seven light intensities.   The data for entire oak and pine seedlings are from KRAMER and DECKER (5).

sumably, the light intensity at the surface of all the needles averages only about 3000 to 3500 fc in full sun and much less in shade.

As the seedlings grow larger mutual shading probably becomes quite important, even in hardwoods, because an increasing proportion of the leaf surface is so located that it is shaded by branches and other leaves.

In view of these results it is possible that completely illuminated pine needles require no higher light intensity for maximum photosynthesis than do hardwoods.   The failure of pine seedlings to carry on photosynthesis in the shade as rapidly as hardwood seedlings is probably the result of greater mutual shading in pine than in hardwoods.   This does not exclude the possi-

bility that other factors contribute to the difference.   It may be that instead of a direct effect of light on photosynthesis there is an indirect effect through the influence of light intensity on stomatal opening.   There might even be a fundamental difference in the photosynthetic mechanism.   It is hoped that these possibilities can be investigated at some future time, but it is believed that they are unlikely to be of major importance.   .

Summary

The rates of apparent photosynthesis of individual, fully exposed, loblolly pine needles were measured at various light intensities and a constant temperature of 25° C.

Fully exposed pine needles reached their maximum rate of photosynthesis at about one-third of full sunlight and showed no further increase in rate up to the highest light intensity to which they were exposed, 9200 fc.   They behaved in essentially the same manner as the hardwood seedlings investigated by KRAMER and DECKER, but differed from entire pine seedlings which attained their maximum rate of photosynthesis at 9300 fc and reached only sixty per cent. of maximum at a light intensity equal to one-third of full sun.

It is believed that the relatively low rate of photosynthesis of entire pine seedlings in the shade results principally from mutual shading of the needles by one another.   Because of the arrangement of the needles, there is much more mutual shading in pine than in hardwood seedlings.

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CHANGES IN FOOD RESERVES AND RESPIRATORY CAPACITY OF BINDWEED TISSUES ACCOMPANYING HERBICIDAL ACTION OF 2,4–DICHLORO–PHENOXYACETIC ACID[1]

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Introduction

Widespread interest in the use of the synthetic growth regulator, 2,4-dichlorophenoxyacetic acid, as an herbicide has emphasized the need for increased knowledge of the mechanism of action of this and similar substances. Furthermore, the special significance of bindweed (*Convolvulus arvensis* L.) as an economic problem and the extensive study of its control by other means (**2, 3, 6, 19**) have made desirable the investigation of herbicidal action of the compound. HAMNER and TUKEY (**8**) and MARTH and MITCHELL (**11**) first investigated the application of 2,4-dichlorophenoxyacetic acid to bindweed, and TUKEY, HAMNER, and IMHOFE (**18**) studied in detail the histological changes preceding death. The most striking responses were the increased meristematic activity in the pericycle, phloem, and cambium regions, and the rapid disappearance of starch granules from the cortex of root and rhizome. An attempt was made in this investigation of the herbicidal action of 2,4-dichlorophenoxyacetic acid on bindweed to correlate changes in food reserves and respiratory capacity of underground structures with the gross symptoms, and the results of the investigation are reported.

Materials and methods

The plants and their treatments were similar to those used in the previous work at this Station (**8, 18**) and the experiments were carried out in the same fields. Three trials were made, two of which were analyzed in detail, the first in July and the second in August, 1945. At least two strips (each 6×1 ft.) of both treated and control plots including tops and underground parts to the depth of about 18 inches were harvested in the early afternoon, wrapped in damp towels, and taken immediately to the laboratory. After washing, the leaves, stems, and underground parts were separated, cut into lengths of $\frac{1}{2}$ to 1 inch, killed in a forced draft oven at 110° C. for 30 minutes, and dried overnight at 70° C. The dried samples were ground to 100-mesh in a Wiley mill and redried at 70° C. immediately before analysis. Aliquots of the fresh samples were dried at 110° C. for moisture determination, and aliquots of each ground sample also were redried at 110° C. for calculation on a dry weight basis.

Carbohydrate analyses were carried out by small-scale modifications of

earlier methods (**5, 9**). The technique, which has not previously been described, was developed primarily for use with tissue slices but was also found suitable for the present material. Samples of 100 mg., containing 1 to 10 mg. of soluble sugars and/or hydrolyzable polysaccharides, were extracted by refluxing for 20 minutes with 20 ml. of 80% ethanol in large test tubes (1 × 8 inch) with cold-finger condensers. The tubes were centrifuged, the supernatants decanted, and the residues reextracted in the same way. Two extractions were found to remove 98% to 99% of the soluble sugars. The combined extracts containing the sugars were concentrated on a steam bath to remove the ethanol and the final aqueous solutions of 4 or 5 ml. transferred to calibrated test tubes. Excess saturated neutral lead acetate was added, the volume made up to 10 ml., and the lead salts centrifuged down. The supernatants were then decanted into other tubes containing excess solid $K_2C_2O_4$ and the PbC_2O_4 centrifuged down. Aliquots of 2 or 4 ml. of the supernatants in large test tubes were acidified to about pH 4 with 10% acetic acid using methyl red; 4 drops of 0.1% invertase (Wallerstein Scales) were added and the tubes incubated 3 to 4 hours at 38° C. The volumes were then made up to 5 ml. with water and the reducing capacities measured with SOMOGYI's new copper-phosphate reagent (**17**) by titration with 0.005 N thiosulphate. The "total sugars" were expressed as mg. of glucose.

The estimation of the "starch-dextrins" fraction of the residues was carried out by a modification of the HASSID, McCREADY, and ROSENFELS technique (**9**) using the copper-phosphate reagent above. It should be noted that grinding beyond 100-mesh or solubilizing by the HCl-ethanol method was not necessary under these conditions. The residues in the original tubes, with 4 ml. of water added, were first heated 30 minutes in a boiling water bath, then 1 ml. of pH 5.6, 0.1 M acetate buffer containing 0.25 N NaCl, 1 ml. filtered saliva (diluted 1:1), and a crystal of thymol were added. The hydrolysis limit was reached after less than 6 hours at 38° C., but the hydrolyses were usually run overnight. The contents of the original tubes were then transferred to calibrated test tubes, clarified, and analyzed as for the soluble sugars. The differences in reducing capacities between samples treated with boiled and unboiled enzyme solutions were expressed as starch based on a Kahlbaum starch standard. These differences were considered a measure of the "starch-dextrins" fraction which appears to be the principal polysaccharide reserve of bindweed roots (**2, 7**). This fraction plus "total sugars" constituted the "available carbohydrates."

Total nitrogen was run on a semi-micro scale by a technique based on that of MA and ZUAZAGA (**10**) using a selenium catalyst. No nitrates were found by the diphenylamine test.

Control and treated samples for respiration measurements from each of three plants were taken from rhizomes 4 to 6 inches below the soil line and from roots 8 to 12 inches lower. Freehand slices 0.3 to 0.5 mm. thick giving 100 to 200 mg. fresh weight per sample were washed in aerated, running tap water for 2 to 3 hours. The rate of oxygen uptake was determined sepa-

rately for root and rhizome slices of each plant in standard Warburg apparatus in 0.02 M pH 5.7 phosphate buffer and an oxygen atmosphere. Under these experimental conditions the rates were linear for at least an hour, were independent of slice thickness and shaking rate, and were proportional to the amount of tissue added. The least significant difference at the 5% level between samples for several experiments similar to that in table I was found to be about 0.10 microliters per hour per mg. fresh weight. Samples of the material from which the slices were cut were fixed and sections prepared as in the previous work (18).

TABLE I

CHANGES IN RESPIRATORY CAPACITY OF RHIZOME AND ROOT SLICES FOLLOWING TREATMENT WITH 2,4-DICHLOROPHENOXYACETIC ACID MEASURED IN WARBURG RESPIROMETER

| DAY | OXYGEN PER HR./MG., FRESH WEIGHT* | | | | | | RATIO OF TREATED TO CONTROL |
| | CONTROL TISSUE | | | TREATED TISSUE | | | |
	RHIZOME	ROOT	MEAN	RHIZOME	ROOT	MEAN	
	$\mu l.$	$\mu l.$	$\mu l.$	$\mu l.$	$\mu l.$	$\mu l.$	
3	0.36	0.32	0.34	0.58	0.39	0.49	1.44
6	0.34	0.27	0.31	0.59	0.57	0.55	1.78
10	0.28	0.31	0.30	0.61	0.64	0.63	2.10
14		.	0.33			0.55	1.67

* Average of three determinations.

Results

The gross response to treatment agreed closely with that observed in 1944 (18) though the sequence of symptoms was somewhat slower. On the second day the leaves were severely folded and most stems were limp. By the fourth or fifth day some lower leaves were chlorotic and a few had necrotic areas. By the sixth or seventh day the rhizomes showed definite external signs of proliferation and both stem and leaves had necrotic areas. By the tenth day swelling of the rhizomes and roots was pronounced and 10% to 20% of the leaves and stems were dead. On the fourteenth day so much of the tops were dead that only root samples were taken for analysis. It should be noted that as far as possible no dead tissue was included in these samples. Histological sections of rhizomes and roots showed similar changes to those reported the previous year (18).

Changes in carbohydrate and nitrogen fractions following treatment in the July samples only are given (table II) since the August samples yielded similar results. Expression on a fresh weight instead of dry weight basis gave the same general picture with somewhat increased ratios of root constituents. However, fresh weight determinations by the tenth and fourteenth days were of limited significance because of wilting in the tops and softening of the root cortex. As early as the second day there was evidence of carbohydrate mobilization in the leaves. Total sugars reached a peak by the fourth day and then returned to the control level while the starch-

TABLE II

CHANGES IN COMPOSITION OF BINDWEED FOLLOWING TREATMENT WITH 2,4-DICHLOROPHENOXYACETIC ACID GIVEN AS PERCENTAGE OF DRY WEIGHT

Day	Tissue	Dry Weight		Total Sugars			Starch-Dextrins			Total Available Carbohydrates			Total Nitrogen		
		C*	T†	C*	T†	T/C‡	C*	T†	T/C‡	C*	T†	T/C‡	C*	T†	T/C‡
		%	%	%	%		%	%		%	%		%	%	
0	Leaf	17.5		4.80			2.55			7.35			4.78		
2		17.7	17.4	5.90	8.15	1.38	4.88	4.45	0.92	10.78	12.60	1.17	4.57	4.80	1.05
4		18.2	17.2	3.60	7.35	2.04	3.18	3.72	1.17	6.78	11.07	1.64	5.08	4.41	0.87
7		17.2	18.6	5.60	8.35	1.49	4.10	2.88	0.70	9.70	11.23	1.16	4.76	4.11	0.86
10		17.0	17.8	3.45	3.65	1.06	3.28	1.18	0.36	6.73	4.83	0.72	5.00	3.31	0.66
0	Stem	17.9		5.70			0.70			6.40			2.25		
2		17.0	17.3	7.35	10.95	1.49	0.60	1.10	1.84	7.95	12.05	1.52	2.23	2.52	1.13
4		19.0	20.2	7.35	11.20	1.52	0.72	1.34	1.86	8.07	12.54	1.56	2.50	2.72	1.09
7		17.7	18.7	6.70	9.80	1.46	0.70	0.80	1.14	7.40	10.60	1.43	2.45	3.19	1.30
10		17.8	18.1	6.75	5.05	0.75	0.87	0.55	0.63	7.62	5.60	0.74	2.53	3.40	1.35
0	Rhizome-root	21.2		6.85			3.48			10.33			1.50		
2		19.4	19.3	6.55	8.20	1.25	5.63	5.65	1.00	12.18	13.58	1.12	1.79	1.60	0.90
4		22.1	19.2	8.15	11.50	1.41	5.31	4.30	0.81	13.64	15.08	1.10	1.73	1.97	1.14
7		19.6	18.3	6.85	11.60	1.70	4.02	2.48	0.62	10.87	14.08	1.30	1.87	2.13	1.14
10		20.9	18.1	6.75	9.45	1.40	6.30	2.45	0.39	13.05	11.90	0.91	1.78	2.48	1.39
14		23.9		6.75	7.65	1.15	6.80	2.25	0.33	13.55	9.90	0.73	1.70	2.67	1.57

* Control.
† Treated.
‡ Ratio of treated to contro

dextrins fraction after a less significant rise dropped to one-third the control level and total nitrogen underwent a steady decrease. Changes in total sugars and starch-dextrins in the stem were similar to those in the leaves though neither the initial increase nor final decrease was so large. The nitrogen content, however, increased significantly above the controls, as would be expected from the histological evidence of meristematic activity in the stem (18). There was, likewise, a rise in total sugars in the roots and rhizomes of treated plants reaching a maximum about the seventh day in this case and again followed by a decrease. The starch-dextrins fraction, on the other hand, steadily dropped after the second day reaching one-third the control level on the fourteenth day. As in the stems the total nitrogen began to increase by the fourth to seventh day paralleling the increasing meristematic activity.

The changes in the carbohydrate and nitrogen fractions in bindweed treated with 2,4-dichlorophenoxyacetic acid were in many ways similar to those observed by MITCHELL and others (1, 13, 14, 15) in kidney bean seedlings treated with indoleacetic acid, naphthaleneacetic acid, and related compounds, and more recently by MITCHELL and BROWN (16) in annual morning-glory treated with 2,4-dichlorophenoxyacetic acid. The initial rise in total sugars in treated leaves before any corresponding drop in the starch-dextrins fraction seemed to indicate a temporary increase of photosynthesis over utilization. The trend was reversed, however, after the first four days and was followed by a drop in both carbohydrate fractions and total nitrogen as the leaves became chlorotic and wilted. If leaf metabolism was stimulated in any way at the outset, it was soon disrupted and became essentially catabolic. On the other hand, the metabolic stimulation of the rhizomes and roots seemed to be essentially anabolic: the histological evidence (18) of meristematic activity and increase in total nitrogen was sharply contrasted with the behavior of the leaves. While leaf reserves were quickly mobilized and translocated or consumed with a decrease in leaf nitrogen content, the mobilization in the rhizome and roots was marked by increased nitrogen content, synthesis of protoplasm, and cell division.

Further evidence of stimulated metabolism in treated rhizomes and roots was apparent in the typical set of respiratory measurements (table I). There was a significant increase in respiratory rates of treated tissues over control tissues as early as the third day in the rhizome and the sixth day in the lower root tissues. The increased ratio reached a maximum by the tenth day and declined by the fourteenth. Histological sections from the same samples, however, showed little if any cell division by the third day, indicating, as might be expected, that increased respiratory activity preceded increased cell division. The drop in rate of treated slices by the fourteenth day was probably due to the already evident disorganization and rupture of the cortical regions.

It is also interesting to compare the results of treatment with 2,4-dichlorophenoxyacetic acid with those of other bindweed control methods which have

often been interpreted in terms of their effect on root reserves. BAKKE, GAESSLER, and LOOMIS (2) concluded that in both chlorate treatment and fallowing the death of the roots was correlated with reduction of these reserves, though it did not occur so rapidly as in the case of 2,4-dichlorophenoxyacetic acid treatment. These treatments also differed from the present in causing no increase either in total sugar or nitrogen fractions or, apparently, in cell division. The workers suggest, however, but without direct evidence, that the rapid reduction in polysaccharide reserves with no corresponding rise in soluble sugars following chlorate treatment was due to stimulated respiration rather than to interference with photosynthesis.

TUKEY, HAMNER, and IMHOFE (18) proposed on the basis of histological changes four possible mechanisms contributing to the herbicidal action of 2,4-dichlorophenoxyacetic acid: (1) chlorophyll depletion in the leaves diminishing food production, (2) phloem proliferation in the vascular bundles interfering with translocation of food, (3) increased respiratory activity depleting food reserves, and (4) disorganization and rupture of rhizome and root cortex leading to invasion of soil pathogens and decay. The present analytical evidence, though admittedly limited, may throw some light on the mechanisms. The first was supported indirectly by the evidence for decline in carbohydrate production after the fourth day as the leaves became chlorotic. Similarly, the observed increases in respiratory capacities of rhizome and root slices tended to support the third mechanism. It seemed clear, however, that the reduction in available carbohydrates whether by diminished production or increased utilization was not the principal cause of death. The reduction was considered neither soon enough nor large enough to account, in itself, for the rapid death of the tops and partial disintegration of the roots by the tenth to fourteenth days. In the case of the second mechanism there was no specific evidence in the analyses for interference with phloem function though the possibility is an intriguing one and is given further consideration later. The fourth mechanism may be of major importance in the ultimate death of underground structures, but it is definitely secondary to the changes investigated and may even be a result of them.

The altered respiratory capacity and increased meristematic activity may be significant clues to other specific physiological abnormalities in the treated plant rather than to simple depletion of food reserves. Several lines of evidence suggest that the phloem may be especially involved: (1) rapid mobilization of food reserves to treated regions, presumably through the phloem, is characteristic of many responses to growth regulators (13, 14); (2) phloem is particularly sensitive to histological reaction to the agents (4, 18); (3) phloem activity in solute translocation (at least in the sieve tubes) is reported to require respiratory energy (12); and (4), it was indicated, though not proved by the study, that the phloem undergoes marked changes in respiratory activity following treatment. One may speculate, therefore, that the treatment caused changes in the respiratory mechanism of phloem

cells which under certain conditions, perhaps low concentrations, accelerated food translocation and under others, perhaps when cell division resulted, impeded it. Whether or not the above hypothesis be true, the analytical results seem to indicate that the herbicidal effect of 2,4-dichlorophenoxyacetic acid on bindweed is probably not due primarily to depletion of food reserves as was the case in earlier methods of control, but is largely the result of other physiological disturbances.

Summary

The mechanism of the herbicidal action of 2,4-dichlorophenoxyacetic acid on bindweed was further investigated by determining the changes in carbohydrate and nitrogen fractions and respiratory capacities of treated and control tissues. Small-scale methods for total sugars and the starch-dextrins fraction were described incidentally.

During the first few days the treatment caused rapid increases in total sugars in leaves, stems, and underground parts, and the increases were followed by decreases to control levels. The starch-dextrins fraction decreased in all three, reaching one-third the control levels in leaves, roots and rhizomes by the tenth day. Total nitrogen, on the other hand, decreased steadily in the leaves but increased in both stems and underground structures. The changes in nitrogen content of treated tissue appeared to be correlated with increased meristematic activity. Further evidence of stimulated metabolism was found in the increased respiratory activities of treated rhizome and root slices.

Comparison of these results with the effect of other growth regulators on bean seedlings showed striking similarities in the general mobilization of carbohydrates and nitrogen. Comparison with the effect of chlorate treatment on bindweed root reserves also showed certain similarities but did not indicate that reserve depletion as such was the chief mechanism of herbicidal action in the hormone treatment.

The bearing of the analytical results on the mechanisms of herbicidal action proposed by TUKEY, HAMNER, and IMHOFE (18) was discussed, and it was suggested that a specific mechanism of herbicidal action of 2,4-dichlorophenoxyacetic acid might be found in the interference with phloem function.

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STUDIES ON THE RELATIONSHIP BETWEEN THE RATE OF INFECTION OF COTTON SEEDLINGS BY *PHYMATOTRICHUM OMNIVORUM* AND THE AVAILABLE OXYGEN SUPPLY[1]

LETA HENDERSON-WEBBER AND HAROLD H. WEBBER

In a previous paper (5) it was suggested that growth of and infection by the cotton root-rot organism, *Phymatotrichum omnivorum,* were both dependent on the oxygen supply available to the organism, since infection did not occur unless the organism was growing near the surface of the medium. The work of MOORE (7) has shown that for agar cultures the optimum oxygen concentration over the culture is that of the atmosphere, or about 21%. When the cultures are submerged, growth is apparently directly correlated with oxygen concentration in the medium. Under ordinary conditions the oxygen concentration in the lower part of a liquid culture approaches zero.

It was noted in previous experiments (5) that floating inocula of *P. omnivorum* grew much more rapidly than did those which were submerged. Infection of cotton seedlings was correlated with these conditions in such a way that when the culture was submerged, there was a delay in infection until the fungus had grown to the surface. Infection by surface cultures was much more rapid than by submerged cultures. Such a correlation led to the hypothesis that the oxygen concentration available to the fungus under these conditions was the limiting factor in infection. Work reported in this contribution was designed to prove this hypothesis.

Methods

The culture media, methods of inoculation, and seed treatment employed have been reported in a previous paper (5). The obvious approach to the problem would be to control the aeration of the culture medium so as to maintain uniform oxygen concentration throughout. It was found that simple aeration devices could not be employed with *P. omnivorum* cultures, since even the smallest bubbles from a sintered glass aerator cause sufficient disturbance of the culture to impair normal growth. The root-rot organism is very sensitive to mechanical disturbance when grown in liquid media, so that the growth rate is lessened considerably by shaking or even handling. It was necessary, therefore, to resort to other devices to accomplish aeration.

The aeration device used is shown in Tube T, figure 1. Half-inch cellophane dialysis tubing was cut into nine-inch lengths. A glass U-tube with an inlet (A) and an outlet (B) aperture was bent as shown. The gas

entered the cellophane bag through opening (F) and returned to the glass tube through opening (D). The outlet (B) opening was reduced in diameter so that a slight pressure would result, thereby maintaining the cellophane bag (C) distended. The dialysis tubing was pushed around the glass tube and sealed by means of household cement (cellulose nitrate). The tubing was long enough so the seals would always be above the level of the
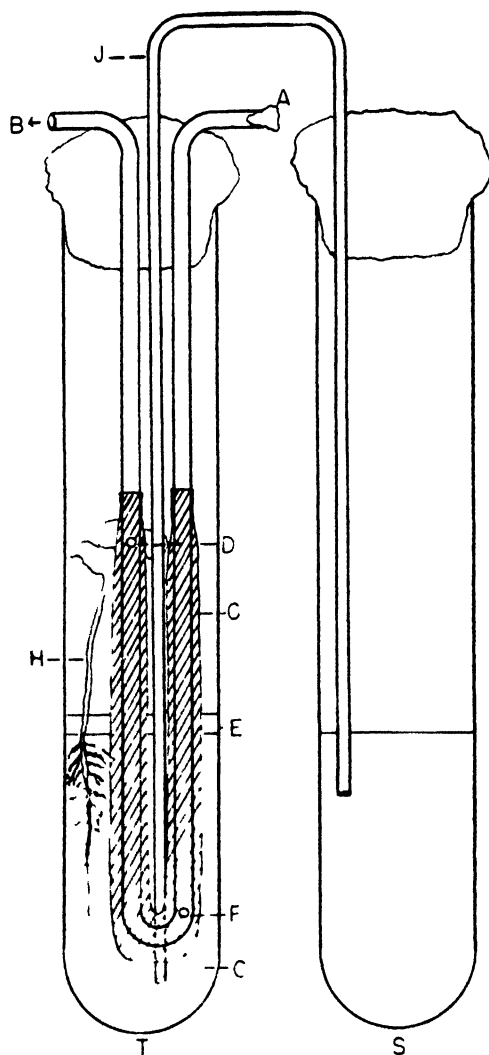
FIG. 1. Apparatus for the double culture of *Phymatotrichum omnivorum* and the cotton plant.

culture solution. An imperfect seal would thus not disturb the culture by allowing bubbles to pass up through it. The opening of the inlet tube was plugged with cotton so the air would be sterilized by filtering before it passed through the bag. The inlet tubes were attached to a source of compressed air, and a pressure of three cm. of mercury was maintained during an experiment. The test tube, with aeration device and medium, was sterilized in the autoclave for 20 minutes at a pressure of 15 pounds. During

sterilization and until the air pressure was applied, the tubes with the cellophane bags were kept above the surface of the medium to prevent the medium from passing inside the bags. When the air was attached, the pressure inside was sufficient to prevent the inward flow of the liquid medium.

The efficiency of the aeration apparatus was tested in order to determine the rate of oxygen absorption by the culture solution. Oxygen dissolved in the culture solution was measured by the WINKLER method. The procedure was modified according to VAN DAM (12). A 5-cc. hypodermic syringe was used as a collecting tube. A 3-inch spinal puncture needle was attached to it by means of sealing wax. The whole collecting device was simple enough for sterilization. In collecting a sample, the syringe with the attached needle was filled with the $MnSO_4$ solution, the plunger was forced completely down so that only the bore of the needle and the space between the wall of the syringe and the plunger were filled with $MnSO_4$. This amount was sufficient for an analysis. The tip of the needle could be easily sterilized, if necessary, so that it could be inserted into a sterile culture without danger of contamination. A sample for analysis was obtained by pushing the plug of the test tube to one side, inserting the syringe and needle so that the tip of the needle reached the bottom of the tube, and withdrawing a 5-cc. sample into the syringe, mixing thoroughly with the $MnSO_4$ during the process. The other reagents were added by drawing them into the syringe and shaking thoroughly. At no time was the sample in direct contact with air. Titration was carried out using N/10 sodium thiosulphate in a 0.2-cc. automatic micro burette.

It was necessary to test several materials as seals (E) against the permeation of oxygen before selecting the conditions for the experiments. Paraffin proved too brittle to allow the penetration of the needle for drawing off the oxygen test sample. Paraffin oil was unsatisfactory due to the solubility of oxygen in paraffin oil. Paraffin-paraffin oil mixtures or paraffin-petrolatum mixtures, however, proved efficient as seals against oxygen. The data from one of these experiments are presented (fig. 2 and table I).

Twelve tubes of mineral solution were set up. The first two were covered with 10 cc. of a mixture containing equal amounts of paraffin, petrolatum, and paraffin oil. The second two tubes were covered with 10 cc. of a mixture containing equal amounts of petrolatum and paraffin oil. The third two tubes were covered with one-half petrolatum and one-half paraffin. Tubes 7 and 8 were similar to 1 and 2 except that they were aerated; tubes 9 and 10 duplicated 3 and 4 but were aerated; tubes 11 and 12 duplicated 5 and 6 but were aerated. After first being sterilized for 20 minutes in an autoclave, all tubes were placed in a water bath at 30° C. Readings were begun as soon as the temperature of the tubes had reached equilibrium with the bath. The results are shown in table I and figure 2.

Any combination of materials used proved to be an adequate seal, as shown by tubes 1, 2, 3, 4, 5, and 6. The cellophane tubing permitted oxygen

TABLE I

EFFECT OF SEAL ON ABSORPTION OF OXYGEN FROM AIR

HOURS	AVERAGE OXYGEN IN SOLUTION					
	PARAFFIN-PETROLA-TUM-PARAFFIN OIL SEAL		PETROLATUM-PARAF-FIN OIL SEAL		PETROLATUM-PARAF-FIN SEAL	
	(TUBES 1 & 2) UNAERATED	(TUBES 7 & 8) AERATED	(TUBES 3 & 4) UNAERATED	(TUBES 9 & 10) AERATED	(TUBES 5 & 6) UNAERATED	(TUBES 11 & 12) AERATED
	%	%	%	%	%	%
0	0.290	0.300	0.291	0.291	0.295	0.304
3	0.310	0.363	0.304	0.342	0.287	0.340
6	0.318	0.397	0.291	0.373	0.295	0.376
11	0.321	0.414	0.300	0.441	0.296	0.423
22	0.335	0.433	0.308	0.461	0.315	0.455

to pass through in a short time, as shown in tubes 7, 8, 9, 10, 11, and 12. All the oxygen in these solutions passed through the cellophane, since the ture of petrolatum and paraffin was easier to manipulate in making the seal and in withdrawing the sample, and since it proved to be as efficient a seal as any, it was used as the seal in all other experiments. Since glucose must solutions were covered with the same seals as the preceding tubes. A mix- be present in the culture medium in relatively high concentration for opti- mum growth of Phymatotrichum, several experiments were conducted to test the oxygen uptake of high glucose media.

The results of all experiments using mineral solution and mineral solu- tion plus glucose have been summarized (table II and fig. 3). It will be noted that the solutions were saturated with oxygen between 12 and 18 hours of aeration. The seal, however, apparently allowed a small amount of oxygen to permeate the solution, but a consistently low level was main-
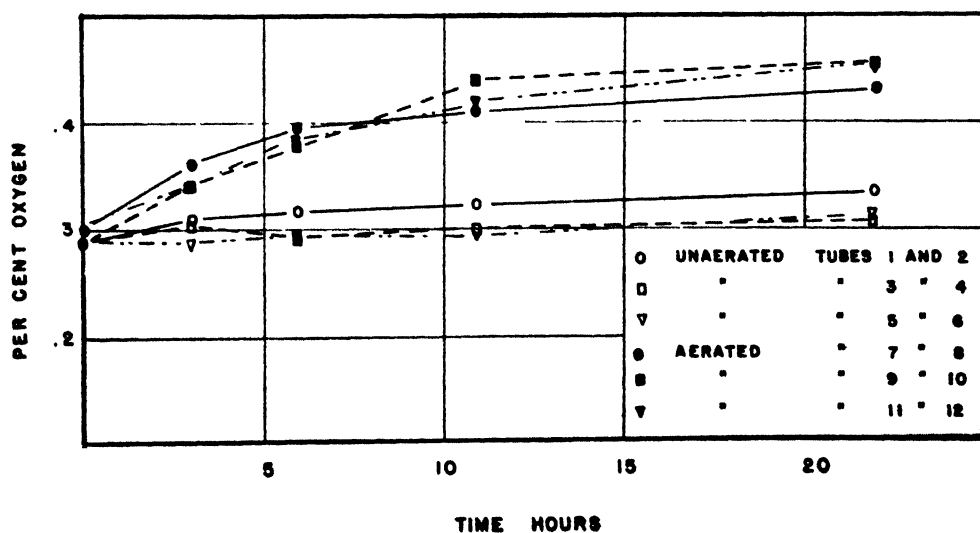
FIG. 2. Effect of seal on absorption of oxygen from air.

TABLE II

OXYGEN UPTAKE IN AERATED AND UNAERATED MINERAL AND MINERAL PLUS GLUCOSE SOLUTIONS

HOURS	OXYGEN IN SOLUTION			
	MINERAL SOLUTION		GLUCOSE SOLUTION	
	UNAERATED	AERATED	UNAERATED	AERATED
	%	%	%	%
0	0.238	0.244	0.164	0.135
2	0.264	0.321	0.170	0.212
4	0.260	0.387	0.175	0.220
8	0.265	0.445	0.185	0.360
12	0.282	0.471	0.195	0.370
24	0.317	0.500	0.190	0.401

tained in the unaerated media. The mineral solution and the sugar solution values for one set of conditions parallel each other closely. The sugar solution, however, is consistently lower in oxygen concentration than the mineral solution. By averaging all the values at any one time for both mineral solution and sugar solution and comparing one with the other, an average difference of 0.10% was obtained. If all the values for the sugar solution (table II) are raised by this amount, the sugar solution curve is very similar in shape and position to the mineral solution curve (fig. 3). This difference is apparently due to the difference between the solubility of oxygen in the mineral salt solution and its solubility in the medium containing 4% glucose. This difference in solubilities is confirmed by published reports (9). The
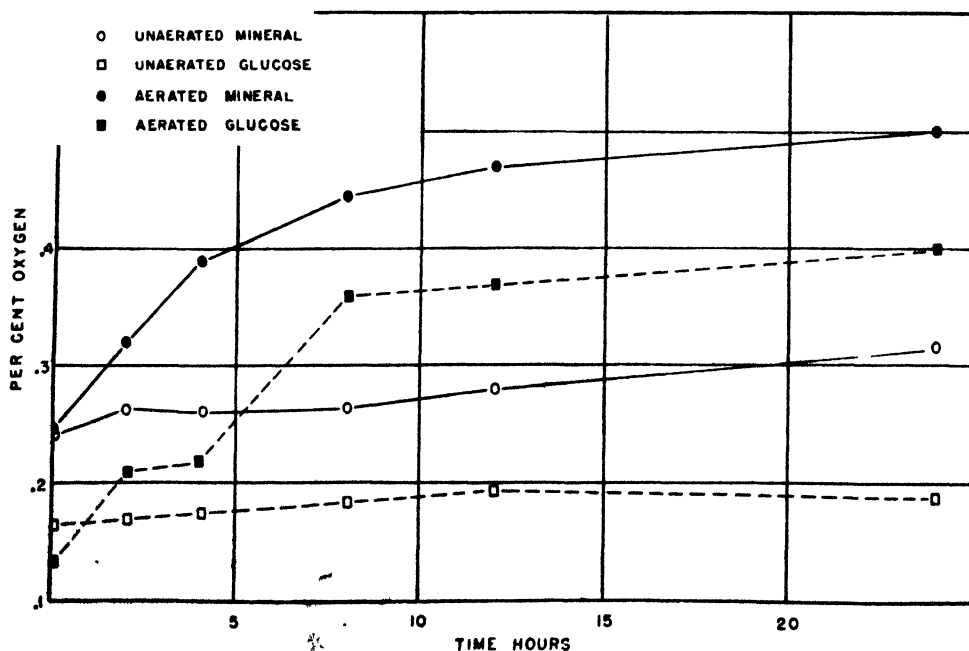
FIG. 3. Oxygen uptake in aerated and unaerated mineral and mineral plus glucose solutions.

values for the amount of dissolved oxygen in the mineral solution and in distilled water when saturation is reaching at 30° C. are comparable with those given in chemical handbooks for amount of oxygen from air dissolved in water.

Experimental results

Large test tubes were inoculated with *P. omnivorum* by transferring 10-cc. aliquots of an actively growing week-old liquid culture into 40 cc. of fresh medium for the test incubation. Group 1 consisted of eight tubes sealed with paraffin-petrolatum and aerated by means of the cellophane bags; group 2 consisted of eight tubes unsealed; and group 3 consisted of eight tubes sealed but not aerated. The tubes were incubated in a water bath at 30° C. for 17 days. At the end of the incubation period the experiment was dismantled and the mass of mycelia from each tube carefully rinsed in distilled water, blotted, and placed in watch glasses. Each lot was dried to constant weight (18 hours at 60° C.) and then weighed (table III).

TABLE III

WEIGHT OF DRIED CULTURE OF *P. omnivorum* GROWN UNDER DIFFERENT
OXYGEN CONCENTRATIONS

GROUP	NUMBER	MEAN WEIGHT	STANDARD DEVIATION
		mg.	
Inoculum control	8	10.7	3.2
1	8	151.6	2.3
2	7	97.9	1.9
3	5	11.4	1.0

The weight of the inoculum was determined by washing, drying, and weighing eight inocula by the same methods as were employed in handling the test cultures.

Similar results were obtained on several repetitions of this experiment. It is apparent that a supply of oxygen is necessary for growth of the fungus. Growth in the sealed tubes was slight, amounting only to 0.7 mg. when the weight of the original inoculum was subtracted from the average of group 3. The slight growth in the sealed, unaerated tubes might have been due to a small amount of oxygen carried in on the inoculum and/or the slight seepage of oxygen through the seal. In the unsealed tubes, growth amounted to 87.2 mg. of dry mycelial weight. This is 125 times more than in the sealed tubes. Oxygen in this case was obtained only at the surface of the cultures by diffusion from the atmosphere. Since the inside diameter of the tubes was 3.6 cm., the surface was 10.17 sq. cm. Growth in the aerated tubes resulted in 141 mg. dry weight of mycelia. This is 1.6 times more than occurred in group 2. The surface of the cellophane bags which were employed in group 1 was approximately 15.5 sq. cm. It appears from this that growth of the fungus is correlated with the area of the membrane, or the

TABLE IV

MEAN WEIGHT OF MYCELIUM PRODUCED IN VESSELS ALLOWING DIFFERENT
AREAS OF EXPOSURE TO AIR

VESSEL		AREA	MEAN WEIGHT	STANDARD DEVIATION
FLASK	TEST TUBES			
cc.		sq. cm.	mg.	
500	....	78.5	59.3	6.6
250	....	51.5	48.2	5.3
125	.. ..	34.2	41.3	6.6
	Large	9.6	27.3	0.0
	Small	3.8	16.6	1.1

effective surface across which oxygen can diffuse. Diffusion of oxygen through liquid cultures is not rapid enough to supply the needs of a rapidly growing submerged culture.

To test the relationship between the surface area exposed to air and the growth of the fungus, an experiment was set up wherein the vessels in which the fungus was grown allowed various surface areas of media to be exposed to atmospheric oxygen. Group 1 consisted of five 500-cc. Erlenmeyer flasks, group 2 of five 250-cc. flasks, group 3 of five 125-cc. flasks, group 4 of five large test tubes, and group 5 of five small test tubes. 40 cc. of medium were placed in each vessel. Each was inoculated with a uniform inoculum from an agar culture of *P. omnivorum*. At the end of a two-week incubation period the fungal mats which had formed on the surface of the liquid media were rinsed in distilled water, blotted, and dried as before. The mean weights of these mats with the area of the vessels in which they were grown are given (table IV and fig. 4).

Since it has been demonstrated that growth of the root-rot organism is dependent on the amount of oxygen available to it, the effect of oxygen
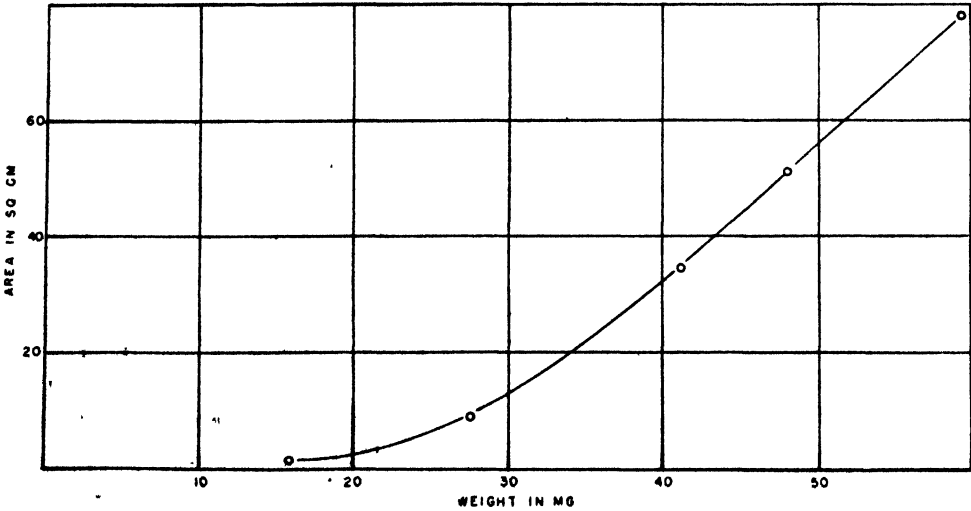
FIG. 4. Mean weight of mycelium produced in vessels allowing different areas of exposure to air.

concentration on the infection by this organism as measured by wilting of cotton seedlings was then studied. A slight modification in the apparatus was necessary since some liquid was removed from under the paraffin-petrolatum seal by the transpiring cotton plant. A second tube (S) was connected to the experimental tube by a siphon (J). As the liquid was removed from the first tube it could be replaced aseptically, and without breaking the seal, by merely raising the second tube to the necessary height. This culture unit is shown (fig. 1). The entire unit was set up complete, except for the seal, and sterilized in the autoclave. Precautions were taken to avoid breaking the siphon during the following manipulations. The experimental tubes were inoculated with uniform-sized inocula from a two-week-old agar culture of *P. omnivorum*. The inocula were purposely placed at the bottom of the tubes. A sterile paraffin-petrolatum seal was poured over the surface of the medium and around the tubing which passed through it. When the seal had solidified, a small opening was made at one side, and

TABLE V
DAYS REQUIRED FOR WILTING OF COTTON SEEDLINGS UNDER DIFFERENT OXYGEN CONCENTRATIONS

GROUP	NUMBER	MEAN NUMBER OF DAYS	STANDARD DEVIATION
Sealed and aerated	8	11.6	2.2
Unsealed	8	14.8	2.9
Sealed and unaerated	8	16.5	2.3

a sterile germinating cottonseed placed in it in such a way that the seed rested on the surface of the seal with the hypocotyl extending down into the medium. A small piece of sterile cotton was wrapped around the hypocotyl at the base of the seed to act as a wick in order that humidity conditions would be favorable for the opening of the cotyledons.

Three groups of cultures were set up: group 1 consisted of eight experimental tubes as just described with the aerating device and siphon; group 2 consisted of eight unsealed and unaerated tubes; and group 3 consisted of eight tubes in which there were no aerating devices, but in which the medium was covered with the seal. There were thus three different oxygen concentrations under which the cotton root-rot organism, and the cotton plant could be grown. The number of days for the various seedlings to wilt as a result of an attack by the fungus was noted (table V).

Isolations of the organism from the infected roots were performed in several cases, but this procedure was unnecessary since in all cases the fungus could be observed in the roots with no difficulty, and wilting was manifest within two or three days after the onset of attack. Since the plants were growing in liquid media and the aerial portions of the plant were growing in an atmosphere of high humidity, wilting was somewhat slower than it would have been in the field or even in potted plants in the greenhouse.

Discussion

The results obtained from the double culture of cotton plants and Phymatotrichum in liquid culture under controlled conditions have enabled the isolation of one factor of the environment for study. It has been demonstrated that cotton seedlings can be killed by the root-rot organism in liquid culture and that infection can be limited by control of oxygen concentration in the medium. The susceptibility of cotton seedlings to the organism under the conditions of the reported experiments indicates little justification for the frequently expressed opinion that young cotton plants are resistant to root rot. BLANK (2) has reported a differential susceptibility to root-rot infection between young and old seedlings and suggested the possibility that carbohydrate accumulation in seedling tissues enhanced the infectivity of the organism. WATKINS (13, 14) employing pure cultures, studied infection of cotton seedlings in media of high carbohydrate levels and demonstrated early kill not only of cotton seedlings but also of "immune" plants, e.g., corn. EATON AND RIGLER (3), however, tested the carbohydrate hypothesis of root-rot infection and concluded that in soil cultures the carbohydrate concentration in root tissue does not affect infection. The Phymatotrichum cultures reported here were grown in media containing four per cent. glucose; therefore, according to BLANK AND WATKINS, conditions for infection were favorable. Under these conditions, it was possible to limit infection rate solely by altering available oxygen; consequently, it may be concluded that oxygen is one of the major factors in growth and infection.

The artificiality of laboratory conditions precludes a ready translation of these results to an interpretation of field observations. However, such an attempt should be made, recognizing the limitations of such an extrapolation. Under field conditions infection of cotton seedlings has been observed infrequently; ROGERS (8) expressed the belief that field kill occurs only in well-advanced plants. Soil temperature and moisture content during the seedling growing period have been suggested as limiting factors in infection as well as the reduced probability of contact between roots and organism at that time of the year.

That oxygen can play an important role in the complexity of factors influencing infection in the laboratory suggests the possibility of a similar effect of oxygen in the field. The high moisture content of the soil in the spring may limit the oxygen supply to rapidly growing organisms in the lower soil strata, since it has been demonstrated (5) that diffusion through a liquid medium is not sufficient to maintain a submerged culture at high infective rate of growth.

STREETS (10) reported that flooding experiments in the Yuma Valley demonstrated a reduction in the severity of the disease, but he was unable to effect an eradication. TAUBENHAUS et al. (11) experimenting with flooding methods in Texas were also unable to eradicate the disease in the field, but found that mycelia survive only a few days in flooded soils in the labora-

tory. These findings may be explained by the results of KING (6), who found that the sclerotia stage of the organism was able to survive long periods of submersion.

Conditions of moisture and aeration in the field change considerably as the growing season progresses. With the drying out of the blackland (Houston and Wilson clay) soils of Texas, aeration is enhanced both by cultivation and the natural fissures that appear in the soil. Fissures serve as deep channels for the transport of gases and provide a means for exchange of oxygen and carbon dioxide at the deeper soil levels (1). The evidence reported here does not support the observation of GILBERT (4) that the fungus seems to grow best, and hence the disease is most severe, when the soil aeration is poorest.

Summary

An apparatus for the double culture of cotton plants and *Phymatotrichum omnivorum* in pure culture is described. Growth of the organism, and consequently infection, are directly correlated with the available oxygen supply. Application of these results to field observations concerning seedling infection and cultural practices is discussed.

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LEVULINS AND INULIN IN GUAYULE, *PARTHENIUM ARGENTATUM* A. GRAY[1]

HAMILTON P. TRAUB AND M. C. SLATTERY
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Recently McRary and Traub (16) isolated a fructosan, or levulosan, tentatively identified as inulin, from guayule plants. Later Hassid *et al.* (11) definitely characterized this fructosan as inulin. Further work on the water soluble reserve carbohydrates of guayule has shown that in addition to inulin, levulins are also present, and in relatively greater amounts. The application of the principles developed in the present paper to physiological research problems by the authors and others (39, 40, 3, 5, 6, 7) has shown that the fractionation of the levulins into 89% ethanol soluble and insoluble portions may be used as a more dynamic tool in plant physiological research than the mere recording of total residues. The preliminary results concerning the free monosaccharides in guayule and the analytical methods for determining the water soluble carbohydrates in guayule are summarized elsewhere (41, 39).

According to McRary and Slattery (15), continuous extraction, for six hours, of the finely ground guayule tissue when 80% ethanol by volume is placed in the boiling flask of the extraction apparatus (Landsiedl type), completely removes the "reducing sugars and sucrose" leaving the "fructosan" in the insoluble residue. Under the conditions indicated, it should be pointed out that the condensate dripping into the extraction cups is approximately 89% ethanol by volume (9) and is approximately at the boiling point of the solvent. In order to show that the interpretation of McRary and Slattery (15) is untenable, previous work on the naturally occurring polysaccharides in some Compositae will be briefly reviewed, the water soluble carbohydrate reserves in guayule will be considered, and the fractions separated from guayule by McRary and Slattery (15) will be critically examined.

Previous work

In 1870, Popp (24) reported a "soluble modification of inulin" in the tubers of *Helianthus tuberosus* which he named "inuloid." Since that date apparently the nature of the inulides (inuloid) or levulins in plants and their relation to inulin, and also the characteristics of inulin as it occurs in nature, were little understood until the subject was elucidated in 1932–1933 by Schlubach and Knoop (30).

Although Tanret (35, 36) in 1893 reported that he was able to separate from inulin associated matter (pseudo-inulin, mol. wt. 2610; inulenin, mol.

wt. 1656; helianthenin, mol. wt. 1924; and synanthrin, mol. wt. 1314) having a lower specific rotation than inulin, neither DEAN (8) nor PRINGSHEIM and ARNOWSKY (26) could confirm these results. ÖHOLM (23) also showed on the basis of diffusion coefficients that the Kahlbaum preparation of inulin he used in his experiments apparently did not contain Tanret's reported synanthrin for the rates of diffusion were in harmony with STEFAN's tables (34). On the assumption that Tanret's interpretation was valid, Öholm observed that the inulin sample apparently was not a pure one since it gave the same diffusion coefficient as TANRET's (35, 36) postulated pseudo-inulin, mol. wt. 2612.

DEAN (8), in 1904, on the basis of work with *Dahlia variabilis, Helianthus tuberosus, etc.,* concluded that "Inulin is accompanied in these places [underground organs] by levulins, resembling inulin in composition, but differing from it in possessing a greater degree of solubility and a lower specific rotation." As a working hypothesis, Dean considered "the carbohydrates or carbohydrate mixture, which is readily precipitated in cold alcohol of 60% strength and has a specific rotation $[a]_D = -33°$ to $-40°$, inulin; and the undetermined mixture of lower rotatory power and greater solubility, the levulin mixture." WILLAMAN (43), in 1922, observed that on the basis of his experiments with *H. tuberosus,* Dean's theory with reference to the identity of inulin which postulates "the existence of various aggregates of molecules, more or less loosely combined; the greater the aggregate, the greater is its specific rotation, and the lower is its solubility in water . . . explains fairly well the behavior of inulin on successive crystallizations, either from water or from alcohol."

On the basis of results reported by SCHMIEDEBERG (31), MEYER (18), and GREEN (10), PRINGSHEIM (31) regards the "inulide which frequently accompanies inulin in nature . . . as a metabolic conversion product of inulin." He regards this fraction as "inulin-dextrins arising as a fermentative intermediate stage in plant metabolism," their occurrence depending on seasonal and other circumstances (25, 27). He cites the observation of POPP (24) that "a fructoside of this nature is polymerized to insoluble inulin on ageing, *e.g.,* in Helianthus nodules." GREEN (10) isolated an enzyme from sprouting girasole tubers, *H. tuberosus,* by extraction with glycerine, that split inulin into "a sugar and an intermediate body possessing properties which resemble those of inulin" but differs from inulin in such particulars as solubility in water and ethanol, and rate of dialysis.

In marked contrast to the conclusions of all previous workers, SCHLUBACH and KNOOP (30) report that the mixture of levulosans in the tubers of *H. tuberosus* apparently consists of three components only: inulin, a dilevan, and another di-D-fructose anhydride. According to these authorities there is no building up of inulin by polymerization of the lower polymer compounds, but each of the three is in turn built up directly from the common fundamental unit, fructose.

Recently McDONALD (17) summarized the knowledge of well-defined

polyfructosans and difructose anhydrides. Consideration of similar or related compounds occurring in plants that are not as yet well defined is omitted but cannot be ignored by the physiologist. According to McDONALD (letter dated March 6, 1946), the "so-called levulins and inulides which occur along with inulin contain glucose as well as fructose, and hence, unless they are well-defined compounds, can only be referred to as polysaccharides." The term, "levulins," therefore, is used to refer in the usual sense to the mixture of non-reducing polysaccharides, in Compositae, containing mostly fructose and lesser proportions of glucose, soluble in cold water and associated with inulin which is not appreciably soluble in cold water.

LEVULINS AND INULIN IN GUAYULE

Using the cold and hot water extraction procedure of TRAUB et al. (**42**), the nature of the water soluble reserve carbohydrates in guayule was first tentatively identified as including cold water soluble levulins in addition to inulin. The fractions will be considered before the sucrose hypothesis of McRARY and SLATTERY (**15**).

INULIN IN GUAYULE TISSUE

Relatively pure inulin was isolated from finely ground guayule tissue according to the hot water extraction with subsequent freezing technique of SPOEHR (**33**). After the precipitation of inulin by freezing, the mass was thawed and inulin recovered by filtering on a Buechner funnel. The product, after three purifications, with an ash content of 0.49 ± .050%, on the basis of three determinations, had a specific rotation of $[a]_D 20 = -37.1°$ ± .17. This compares with $[a]_D 20 = -39.9°$ reported for *H. tuberosus* inulin by SCHLUBACH and KNOOP (**30**), $[a]_D 17 = -36.55°$ for inulin, source not indicated, by BERGMANN and KNEHE (**4**) and $[a]_D = -35°$ for guayule inulin, with an ash content of 1.2%, by HASSID et al. (**11**). The somewhat lower specific rotation, reported by HASSID et al. (**11**), apparently is due to the presence of some of the 89% ethanol insoluble levulins that were extracted with hot water along with the inulin, and were precipitated with the concentration of acetone used to bring down the inulin. The presence of these non-inulin substances in the inulin sample might also affect slightly the other properties reported for guayule inulin by the workers. SCHLUBACH and KNOOP (**30**) have discussed the difficulties encountered in the preparation of relatively pure inulin.

LEVULINS IN GUAYULE TISSUE

Levulins in the finely ground guayule tissue were separated quantitatively from inulin by extraction with water at room temperature, 23° C., (**39**), for at 20° C., water dissolves only 1 part of inulin in 10,000 (**19**). Analyses for total ketoses on the original filtrate from the Spoehr freezing method (**33**), and on the cold water extracts, after clarification in the usual

manner (**39**), and subsequent hydrolysis with 1% HCl, gave reducing values for ketoses of over 95% of the total non-reducing substances present.   The cold water soluble polysaccharides in guayule tissue may consist mainly of difructose anhydrides or polyfructosans.   If sucrose be present, it would constitute a minor fraction of the whole.   Work reported elsewhere (**39, 40**) shows that the values for levulins in guayule tissue are greater than those for inulin.   The reserve polysaccharides in guayule from the standpoint of solubility in cold and hot water therefore are similar to those found in the tubers of the girasole, *H. tuberosus*, also a Composite, as previously reported by WILLAMAN (**43**) and TRAUB *et al.* (**42**).

The final identification of the components of the levulins fraction in guayule tissue presents a major chemical research problem, and remains to be carried out.   It was possible, however, to conduct preliminary experiments for the purpose of determining whether the components of the levulins fraction form an homologous series of polymers intermediate between inulin and the monosaccharides present in the tissues as postulated by PRINGSHEIM *et al.* (**25**) and others; or, whether the conclusions of SCHLUBACH and KNOOP (**30**), based on *H. tuberosus*, apply to guayule tissue.   The latter authors report the presence of only two difructose compounds, dilevan and another difructose anhydride in addition to inulin in the tubers of *H. tuberosus*. The subject may be explored in a preliminary way by determining diffusion coefficients and molecular weights of the levulins extracted with various concentrations of ethanol.

ÖHOLM (**22, 23**) has pointed out that the approximate molecular weights of compounds, including non-electrolytes, may be calculated from diffusion coefficients, and that the method is applicable in cases where the freezing point depression method does not apply, in the case of colloids for instance, where the presence of slight impurities of electrolytes would give erroneous results.   MEYER (**19**) has emphasized the adequacy of such approximate values in cases where relative ones will suffice.

The approximate molecular weights of the levulins fractions extracted with various concentrations of ethanol were determined by the diffusion method, using the apparatus and procedure described by ÖHOLM (**21**). Guayule tissue, prepared as described by TRAUB (**37**), was first extracted continuously for six hours in a reflux (Landsiedl type) apparatus by placing 100% ethanol in the boiling flask, in order to remove the free sugars.   The extract was analyzed for free-reducing and non-reducing substances (table I).   Since the tissue contained a slight amount of moisture, the condensate after extraction began was 97% ethanol by volume, and if present, any sucrose (approximately 0.59% soluble in 100% ethanol at room temperature), as well as levulins, was extracted.   The residue was then extracted successively with 92%, 89%, and 50% ethanol by volume.   For this purpose the required concentrations of ethanol, from EVANS' table (**9**) were placed in the boiling flasks.   The final residue, after extraction with the ethanol-water mixtures, was then successively extracted with cold and hot water (nos. 5

and 6 in table I). All extractions were made in the presence of calcium carbonate to prevent hydrolysis of polysaccharides. In the case of extracts nos. 2, 3, and 4, the ethanol was evaporated, and the residue taken up in water (table I). Proteins, pectins, tannins, *etc.*, were precipitated from all of the extracts with neutral lead acetate, and the solutions were deleaded and clarified. Aliquots of the clarified extracts, 10-ml., were then introduced into the diffusion apparatus for the determination of the actual amounts diffused at constant temperature, 22° C., in the four aqueous layers of uniform depth. The levulins in the original extracts (nos. 2, 3, 4, and 5, table I), and in the aqueous layers from the diffusion apparatus at the end of the diffusion periods, were determined after hydrolysis as

TABLE I

APPROXIMATE DIFFUSION COEFFICIENTS (D_{22}) AND MOLECULAR WEIGHTS FOR LEVULINS FRACTIONS EXTRACTED FROM GUAYULE TISSUES CONTAINING 13.1% POLYSACCHARIDES: 12.6% LEVULINS AND 0.5% INULIN

FRACTION NUMBER	ETHANOL EXTRACT*		WATER EXTRACT		DRY WEIGHT OF TISSUE		D_{22}	MOL. WEIGHT*
	%	FRACTION NO. RESIDUE	TEMPERATURE	FRACTION NO. RESIDUE	LEVULINS†	INULIN†		
			C.		%	%		
1	97				2.9‡	0.0	‡	‡
2	92	1			3.5	0.0	0.328	521
3	89	2		.	0.7	0.0	0.334	503
4	50	3			5.5	0.0	0.249	905
5			20	4	Trace	0.0		
6			100	5	0.0	0.5	§	§

* The results are reproducible within 8% of the values indicated. The difference between nos. 2 and 3 is therefore not significant, but the difference of either no. 2 or no. 3 as contrasted with no. 4 is significant.

† Expressed as fructose. Similar values for a tissue containing 4.74% levulins, and 3.89% inulin, are: (no. 1) 1.16; (no. 2) 1.20; (no. 3) 0.27; (no. 4) 3.89; (no. 5) trace; and (no. 6) 3.89.

‡ The total 97% ethanol extract contained 3.2% carbohydrates, including 0.3% free sugars, which was subtracted. Due to the presence of free sugars it was not practicable to determine the diffusion coefficient and molecular weight for non-reducing substances in this fraction.

§ Compare mol. wt. of 5000 for inulin reported by HAWORTH *et al.* (12).

total reducing substances expressed as fructose by the SOMOGYI method (32). The relative distribution of the levulins in the four aqueous layers was calculated from the actual amounts present, and the diffusion coefficients were obtained by reference to diffusion tables of SCHEFFER (29) and KAWALKI (14) as compiled by ARNDT (2). Molecular weights were calculated from the following equation: $\sqrt{M} = \dfrac{7 \times 1.07}{D_{22}}$. Results from a typical experiment are given (table I).

HAWORTH, HIRST AND PERCIVAL (12) determined the end groups of inulin as 1,3,4,6-tetramethyl-fructopyranose, and concluded that the inulin mole-

cule consists of a chain having a minimum average length of 30 D-fructose units, and a molecular weight of about 5000. The levulins fractions obtained from guayule tissue by extraction with various concentrations of ethanol have lower molecular weights than that reported for inulin (table I). The results also indicate that the polysaccharide components may be approximately separated into two major parts by extraction of guayule tissue with 89% ethanol, under the conditions indicated, and that the 89% ethanol soluble levulins have lower molecular weights than those which are insoluble in the same solvent. Apparently, these results do not corroborate the hypothesis of PRINGSHEIM (25), and others, for an homologous series of inulides or levulins between inulin and the monosaccharides, nor do they indicate that difructose anhydrides are components of the guayule levulins fraction. Only further research can throw additional light on this subject.

HYDROLYSIS OF LEVULINS

After evaporating the ethanol, McRARY and SLATTERY (15) subjected the residues from 89% ethanol soluble extracts of guayule tissue to hydrolysis with bottom invertase, and after subtracting the values for free-reducing substances from those of the total 89% ethanol soluble carbohydrates, reported the difference as "invert sugar from sucrose." In the experiment, however, it was found that hydrolysis of the 89% ethanol extract residue from guayule tissue with top invertase, or with 1% HCl, gave reducing values very much greater than were obtained with bottom invertase (table II). Hydrolysis with top invertase gave values ranging from 30% to 65% higher than those obtained with invertase of bottom fermentation. The corresponding values for 1% HCl are slightly higher than those indicated for top invertase. It may be of interest to note that ADAMS, RICHTMYER and HUDSON (1) report that the pH conditions for the maximum hydrolysis of sucrose, raffinose and inulin differ, and conclude that a specific inulase rather than a β-D-fructofuranosidase is responsible for inulin hydrolysis

TABLE II

COMPARISON OF EFFECTIVENESS OF BOTTOM AND TOP INVERTASES AND 1% HCl IN HYDROLYZING NON-REDUCING SUBSTANCES FROM RESIDUE OF 89% ETHANOL EXTRACT OF GUAYULE TISSUE

SAMPLE NUMBER	NON-REDUCING SUBSTANCES*			INCREASE, TOP OVER BOTTOM INVERTASE
	HYDROLYSIS			
	BOTTOM INVERTASE	TOP INVERTASE	1% HCl	
	%	%	%	%
1	4.91	7.84	8.23	59.7
2	4.29	7.09	7.86	65.3
3	4.67	7.59	8.04	62.5
4	2.03	2.64	2.79	30.0

* Results expressed on basis of total dry weight of tissue.

when yeast invertases are used. On the basis of the data presented, it is safe to conclude that the major part of the 89% ethanol soluble polysaccharides in guayule is not sucrose, for the use of bottom and top invertases, under the usual procedure for the hydrolysis of sucrose resulted in only partial hydrolysis of the total present in the tissues.

It should be noted that when the solutions of the 89% ethanol soluble residues were hydrolyzed with 1% HCl, the values for ketoses by Roe's method (28) as modified by McRary and Slattery (15) ranged from about 94% to 96% of the total 89% ethanol soluble non-reducing substances (table III). Similar values for fructose by Nyns' method (20) as modified by

TABLE III

PROPORTION OF KETOSES TO TOTAL NON-REDUCING SUBSTANCES. (RESIDUE OF 89% ETHANOL EXTRACT HYDROLYZED WITH 1% HCl)

SAMPLE NUMBER	NON-REDUCING SUBSTANCES*	KETOSES*†	PROPORTION OF KETOSES TO NON-REDUCING SUBSTANCES‡
	%	%	%
1	8.23	7.76	94.3
2	7.86	7.51	95.5
3	8.04	7.79	96.9
4	2.78	2.64	95.0

* Expressed on basis of total dry weight.
† Total ketoses by Roe's method (28) as modified by McRary and Slattery (15), less fructose of free sugars by Nyns' method (20) as modified by Jackson and Mathews (13). Ketose values include any ketoses, other than fructose, if such constitute a portion of the total free sugars Traub, Slattery and Walter (41).
‡ Expressed on basis of total non-reducing substances.

Jackson and Mathews (13) agreed with those for total ketoses. Again sucrose as the major polysaccharide originally present in the tissues is ruled out. The relatively high ketose values point to levulins as the water soluble polysaccharides associated with inulin in guayule tissues. The 89% ethanol extract, however, apparently represents only part of the total levulins (table I). McRary and Slattery (15) are therefore not justified in assuming that extraction with 89% ethanol under the conditions stated is a valid criterion for the quantitative separation of "fructosan" from some other non-reducing substance in guayule tissues.

FRACTIONATION OF LEVULINS FOR PHYSIOLOGICAL STUDIES

It has already been noted in the discussion of the results reported in table I that extraction of guayule tissues with 89% ethanol effectively separates the levulins into two major portions. The use of this procedure may therefore serve as a convenient sieve to distinguish changes in the two fractions with time. On successive dates such changes may be determined and correlated with such environmental factors as available mineral nutrients, light intensity and duration, CO_2 content of the air, or with low temperature, soil moisture stress (drought), *etc.* Experiments have shown that

the absolute and relative proportions of the two levulins fractions in guayule plants are markedly affected by low temperature, high soil moisture stress, and other environmental factors (3, 5, 6, 7, 38, 39, 40). The making of such measurements has proved to be a more dynamic, and therefore a more valuable tool in physiological research than the analysis of total cold water soluble polysaccharides alone.

ROLE OF CARBOHYDRATE FRACTIONS

On the basis of the available facts, inulin, insoluble in water at ordinary temperatures, is the least available storage form of carbohydrates in the guayule plant, while levulins, soluble in water at ordinary temperatures, are the more readily available reserve carbohydrates in solution in the cell sap. Fructose, or levulose, and other free monosaccharides (7, 39, 41), apparently are the chief carbohydrates of translocation since they are always present in relatively small amounts in the stems and roots of the guayule plant, but predominate in the leaves and inflorescences. However, there is the possibility that levulins may also be translocated.

Summary

1. The presence of levulins in guayule tissues in addition to inulin previously reported, and in relatively greater amounts, has been established. Thus the McRary-Slattery assumption that sucrose is a major reserve carbohydrate associated with inulin in guayule tissues has been shown to be untenable. If sucrose should be present at all, it would constitute only a minor fraction of the total carbohydrate reserves.

2. The levulins may be fractionated into two major portions by extraction of guayule tissues with 89% ethanol: (a) the 89% ethanol soluble fraction, and (b) the 89% ethanol insoluble fraction. On the basis of this fact, a method has been devised for use in seasonal physiological studies to determine the absolute and relative proportions of the two fractions in response to environmental factors. The method is a more dynamic tool for such studies than the mere recording of total residues.

3. On the basis of diffusion coefficients, it has been shown, on the one hand, that Pringsheim's hypothesis for the presence of an homologous series of polymers between inulin and the monosaccharides does not hold for guayule; and on the other hand, that apparently difructose anhydrides are not present in guayule plants although reported in girasole tubers, *Helianthus tuberosus*, also a Composite, by Schlubach and Knoop.

4. The levulins are the chief reserve carbohydrates in the guayule plant, and inulin is secondary in importance from this standpoint.

5. Although, under the experimental conditions, top invertase hydrolyzed from 30% to 65% more of the levulins extracted from guayule tissues than were hydrolyzed by invertase of bottom fermentation, hydrolysis with 1% HCl was required to obtain complete hydrolysis of the levulins.

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AN AUTOMATIC APPARATUS TO COVER AND UNCOVER PLANTS IN PHOTOPERIOD EXPERIMENTS

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In photoperiod experiments short-day plants are usually either covered with cloth shades hung over some sort of framework or else placed in light-
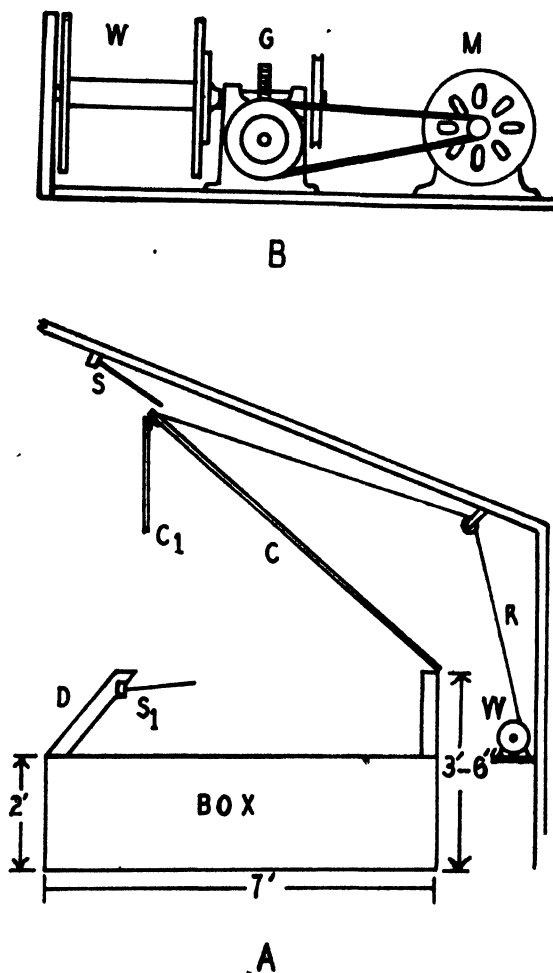
FIG. 1. (A) Diagrammatic end view showing arrangement of box and winch in relation to side wall and roof of greenhouse. (B) Arrangement of motor, speed reducing gear, and winch. The drum of the winch is a piece of hard wood with the outer end turned down to form a shaft. This is supported in a hole in a board attached to the base and lubricated with oil and graphite. The ends of the drum are discs of plywood, and the inner end is bolted to a plate on the reducing gear.

proof boxes. Whatever the details, these methods require someone to be present at a specific time each morning and evening to attend to the plants. Difficulty in finding anyone to do this led to the development of a mechanical

apparatus to cover and uncover groups of plants at any predetermined time, without requiring the presence of an attendant.

The apparatus consists of a box-like enclosure seven feet square, with front and sides two feet high and a back three and one-half feet high (fig. 1). The cover is a light wooden frame (C) hinged to the back and covered with light-proof cloth which hangs down on the sides and front overlapping the wooden sides of the box and darkening the interior. The part of the cover overhanging the front (C$_1$) is mounted on a framework hinged to the top frame allowing it to swing back against the top when the cover is raised, thereby reducing shading of the interior of the box. The cloth forming the sides is attached to the front of the cover and to the back of the box so that the cloth is firmly held in place when the cover is down. Overhead clearance of about 10 feet is desirable to permit raising the cover almost to the vertical position during the day to prevent shading the plants. When the cover is lowered the hinged front slides down the sloping posts (D) at the front corners of the box. This prevents it from being caught on the posts or walls in such a way as to prevent complete closure. If the box faces approximately south, the cover can be pulled back toward the north where it intercepts the minimum of light.

Boxes of various sizes can be constructed. Larger boxes should be rectangular with the cover hinged on the long edge as too much head room is required for a cover larger than six or seven feet wide. Under some conditions a ventilator fan would be necessary to prevent the temperature inside the closed box from becoming much higher than outside. This can be controlled by a switch operating only when the cover is down.

The position of the cover is controlled by an electric time switch through which current is supplied to a reversible electric motor (M). The motor drives a 48 to 1 reduction gear (G) to which is attached a small wooden winch (W). The winch raises and lowers the cover by winding and unwinding a rope (R) extending from the cover over a pulley attached to the greenhouse roof and down to the winch. The time switch has two separate circuits, controlled independently, one to raise the cover in the morning, the other to lower it at night. One of the circuits operates a double-pole double-throw relay used as a reversing switch, so the motor can be operated in one direction to lower the cover and in the opposite direction to raise it. Limit switches (S and S$_1$) are so placed that when the cover reaches the fully open and fully closed positions it operates the switches, breaking the circuit and stopping the motor. Limit switch S$_1$ is placed on the inside of post D and can be operated by a wire projecting from the cover.

A motor of one-sixth horsepower is used in this apparatus, but a one-eighth horsepower motor could lift a cover of the same size. The reduction gear is of the small worm gear type commonly listed in catalogs of scientific apparatus. The limit switches are small toggle switches with extensions made of copper tubing fastened to the metal toggles. They are held in the closed position by gravity and springs except when the cover is pressed

against the toggle extensions. The time clock controls the current from the line to one side of the motor through two separate circuits. One circuit (A) is used to supply current in the morning to lift the cover, the other circuit (B) supplies current to replace the cover in the evening. The sequence of events is as follows: in the morning the time clock closes circuit A, the motor starts, and the cover is lifted until it strikes the limit switch S, stopping the motor. In a few minutes the time clock turns off the current in circuit A. Removal of the cover has meanwhile allowed switch S_1 to close, making circuit B available for use in the evening. At the desired time in the evening the time switch turns current on in circuit B, actuating the reversing switch and running the motor and winch in reverse to lower the cover. As the cover is lowered switch S closes so that circuit A is ready for operation the next morning. When the cover is in the closed position it opens switch S_1 stopping the motor.

Many of the details of this apparatus are susceptible to variation, and ingenious readers will doubtless think of various improvements. The apparatus as described has been in operation over a year with only minor mishaps and has not failed once in the past six months. It provides a practical and convenient method of growing plants with short photoperiods for research and class use.

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NOTES

John Wesley Shive.—Dr. Shive has retired from active professional work this year. He relinquished his duties both at Rutgers University where he has been Head of the Botany and Plant Pathology Department since 1924 and at the New Jersey Experiment Station in Brunswick where he has been Head of the Department of Plant Physiology.

During the thirty-one years Dr. Shive has been at Rutgers University and at the New Jersey Experimental Station, he has been an outstanding teacher of plant physiology. He is an inspiring and conscientious instructor as evidenced by the great number of his students who have continued in teaching and research in plant physiology and related fields.

Dr. Shive is an analyst by nature and is extremely skillful in developing a productive research. He is a scholarly thinker and a sound logician, readily detecting fallacies in unwarranted conclusions. He himself has unusual ability and enthusiasm for research work, and he has been quick to recognize and encourage the efforts and contributions of his students. He has directly supervised the research projects of over fifty graduate students. In addition to the help he has given students in publishing their scientific papers, he also is the joint author of fifty-two papers and the sole author of some forty independent scientific works.

John Shive was born in Halifax, Pennsylvania, in 1877. He received his Ph.B. in 1906 and his A.M. in 1908, both from Dickinson College. From 1913 to 1915 he was a fellow in plant physiology at Johns Hopkins, and in 1915 he received his Ph.D. degree from that institution.

During his professional career, Dr. Shive has achieved international distinction and enjoys a position of highest esteem among his American colleagues. He collaborated with a special Committee on the Division of Biology and Agriculture of the National Research Council under the chairmanship of Dr. B. E. Livingston in formulating and testing standard series of nutrient solutions for plant culture. The comprehensive report of the Committee was published in Baltimore in 1919 as "A Plan for Cooperative Research on the Salt Requirements of Representative Agricultural Plants." He is Associate Editor of *Soil Science*, journal of the Torrey Botanical Club, and a member of the Editorial Board of *Plant Physiology*; he is a charter member and past president of the American Society of Plant Physiologists. In 1938 the latter organization awarded him the honorary Stephen Hales Prize "in recognition of a lifetime of distinguished service to plant physiology." He has been cited and elected to membership in the Royal Agricultural Society of Sweden and in 1938 he was starred in "American Men of Science." He is a member of Torrey Botanical Club and the Botanical Society of America; and he has been elected to honorary membership by the Rutgers Chapters of Phi Beta Kappa and Sigma Xi, serving as Chapter president of each, and of Alpha Zeta.

Dr. Shive's interests and abilities are varied. Not only is he an expert scientist; he is also a photographer of professional grade, a skillful crafts-man of great originality in experimental work, an outdoor enthusiast, and a participant in numerous sports. The American Society of Plant Physiolo-gists and its journal, *Plant Physiology*, are pleased to acknowledge his dis-tinguished contributions as a scientist, as an officer of many years of able service, and as a loyal friend of the profession.

Joseph H. Gourley.—Dr. Gourley, Head of the Department of Horti-culture at the Ohio Agricultural Station since 1921 and Chairman of the Department of Horticulture and Forestry at the Ohio State University since 1929, died at the age of 63 on October 27, 1946, at Wooster, Ohio, following an attack of coronary thrombosis. Until a week before his death he was carrying heavy administrative responsibilities, in addition to the direction of a large class of senior and graduate students and the instruction of 165 freshman majors in horticulture.

The entire field of plant science suffers a great loss in the death of Dr. Gourley, for in addition to outstanding contributions to research in his own field, such as his orchard soil management studies, he possessed a broad knowledge and a stimulating interest in plant physiology and plant anatomy, the subject in which he obtained his doctor's degree at the University of Chicago. He believed deeply in the establishment and maintenance of the closest possible relations between the various fields of plant science, and he felt that horticulture could be adequately served only so long as its investi-gators and teachers possessed thorough basic training in botany and plant physiology. This breadth of viewpoint made him keenly aware of new developments throughout the field so that men doing research under him were always encouraged to advance along new and exploratory lines. Dr. Gourley, who was well known throughout the country, will be greatly missed by all who knew him, not only as a scientist and teacher, but also as a person of warmth and charm with an unlimited capacity for friendship.

Bayard F. Floyd.—On November 8, 1945, Mr. Floyd, Vice-President of the Wilson and Toomer Fertilizer Company of Davenport, Florida, died of a cerebral hemorrhage. He was stricken while attending an all-day meet-ing of the Florida Agricultural Research Institute in Winter Haven, Florida. He is survived by his widow, Mrs. Dixie W. Floyd.

Walter S. Clark.—Probably the youngest member of the American So-ciety of Plant Physiologists, Mr. Walter S. Clark died on October 31, 1946. During the past three and one-half years he has been assisting in the Depart-ment of Botany at Duke University. His exceptional interest and outstand-ing ability in science gained him an early admittance to the University be-

fore his high school graduation. He has published one paper and was co-author of another which is now in press. His high scholarship was recognized in his junior year by his election to Phi Beta Kappa. MR. CLARK is survived by his mother, MRS. FRANCES CLARK BRITTON, of Miami, Florida.

Life Membership.—The Executive Secretary-Treasurer has announced the purchase of a Life Membership by DR. WENDELL R. MULLISON. DR. MULLISON, recently returned from Curacao, is now Plant Physiologist with the Dow Chemical Company at Midland, Michigan. The Society is very happy to welcome DR. MULLISON to the growing roster of Life Members.

The Society offers a Life Membership on the payment of One Hundred Dollars in a lump sum. Life members pay no further dues and they receive the journal for life without any additional charge. Members may become Patrons of the Society upon payment of Two Hundred Dollars (or more at their option) and also receive the journal for life without further cost. A Life Membership represents a sound financial investment as well as the satisfaction of professional distinction.

Crop Production and Environment.—R. O. WHYTE. Faber and Faber, Ltd., 24 Russell Square, London, W. C. 1, England. Ryerson Press, Agent, 299 Queen Street, West Toronto (2B), Canada. 372 pages. $5.00.

The author's long association with the Imperial Bureau of Pastures and Forage Crops at Aberystwyth, Wales, makes him well qualified to undertake a correlation of research in pure and applied science as related to crop production. The subject is approached from the standpoint of development. The terms *growth* and *development* are differentiated on the basis of definitions given at the outset by the author. The first portion of the book critically reviews recent important researches on environmental factors in relation to growth and development, especially as they influence growth substances and nutrients as autogenous factors in ontogeny. The final chapters are devoted primarily to a consideration of the application of the foregoing physiological principles to the production of particular crops. Special attention is given to physiological aspects of the geographical distribution of crop plants, plant introduction, and breeding. The book concludes with a survey of the developmental behavior of crops and methods of manipulation of their growth and development.

The comprehensiveness of the world literature reviewed is correlated in an exceptionally effective manner and makes the book particularly valuable. Despite the handicaps of war, the author appears to have had access to recent publications of many nations and thus brings the discussions up to the present date. Readers will find the thorough appraisal of recent Russian researches particularly illuminating. The problems of production of specific crops are of as much interest to plant physiologists as to agronomists. The historical review of physiological research on growth and development

beginning with the work of Klebs vividly reveals the epoch-making strides made in the brief period of less than three decades. These studies are still gaining in momentum and pace, suggesting that even greater success in understanding and control of life processes in plants may be expected within the next few years.

The subject matter of the book is arranged as a very logical progression of ideas. Figures and tables have been judiciously selected and contribute considerably to clarification of the discussions. Particular attention has been given to citations and indexes which facilitate use of the book for rapid reference. Citations are grouped by chapters but are supplemented by separate volume indexes of authors, genera, and subjects. A very helpful glossary of scientific terms is also included.

Ernährung und Stoffwechsel der Pflanzen. — A. FREY-WISSLING. Büchergilde Gutenberg, Zurich, Switzerland. G. E. Stechert & Company, 31 East 10th Street, New York 3, New York. 295 pages. $2.50.

Plant scientists will welcome this authoritative monograph of plant nutrition and metabolism. The content is grouped under the following major headings: Resorption, Assimilation, Translocation and Accumulation, Growth, Dissimilation, and Elimination. Though the text presupposes a knowledge of physical and chemical fundamentals, it is not extremely technical. The author brings up-to-date the established concepts concerning the topics discussed and includes reference to much recent European research which has been relatively inaccessible on account of the War. In view of the wartime difficulties under which the book was written, it is excellently documented.

With the exception of the comparatively brief discussion of recent work on photosynthesis, all topics are rather comprehensively presented. The author's special competence concerning the physiology of elimination by plants makes this section of the book a very helpful and critical summary. The section on translocation emphasizes important recent researches on the conduction of water and solutes through cell walls. The work of Wieland and Warburg on respiration is reconciled in a clear and concise manner. The book has an attractive and very readable format. It has separate author and subject-matter indexes, the latter being quite comprehensive and thoroughly cross-indexed. Numerous figures and tables contribute considerably to the clarity of the contents.

Contribution to the Physics of Cellulose Fibres.—P. H. HERMANS. Elsevier Publishing Company, New York, New York. 225 pages. $4.00.

. This book on sorption, density, refractive power, and orientation of cellulose fibers constitutes communication no. 21 of the Institute for Cellulose Research of the Algemeene Kunstzijde Unie (A.K.U.) of Utrecht, Holland, and is one of the monograph series, Progress of Research in Holland during the War. Though endeavoring to assemble the results of earlier

related research, much of this publication is an original contribution on the physical properties of cellulose.

Though the subjects discussed are of interest primarily to those engaged in cellulose and fiber research, the interpretations of density measurements and optical properties make the subject matter of value to plant scientists. A special section on experimental methods is included. The differences between native and regenerated fibers in sorptive properties and swelling capacity are given special emphasis. The various sections on the theoretical background of cellulose physics are each concluded by a very concise and useful summary. Though the book lacks a general index, the table of contents is sufficiently detailed to permit use for reference purposes.

Goethe's Botany; The Metamorphosis of Plants (1790) and Tobler's Ode to Nature (1782).—Edited by FRANS VERDOORN. Chronica Botanica Company, Waltham, Massachusetts. 63 pages. $2.00.

Botanists will welcome the translation into English of Goethe's Metamorphosis of Plants and the accompanying critique of his concepts by a very competent reviewer. The origin of Goethe's interest in plants and his general viewpoint of metamorphosis are of special interest. This concise review is authentically documented and written in a very lucid style.

pH and Plants.—JAMES SMALL. Baillière, Tindall and Cox, 7 and 8 Henrietta Street, Covent Garden, London, England; G. E. Stechert & Co., 31 East 10th Street, New York 3, New York. 216 pages. $4.00.

This compact book reviews recent important methods and data on pH in relation to plant physiology. Methods of approach, applications, and types of results expounded provide a helpful résumé of the current status of hydrion studies. A wide variety of data are included on interrelationships between pH and such topics as metabolism, enzymatic reactions, plant structures, pathology, and ecology. The book is well illustrated, and a selected bibliography of over 300 important papers is included.

EFFECTS OF NITROGEN ON CHLOROPHYLL, ACIDITY, ASCORBIC ACID, AND CARBOHYDRATE FRACTIONS OF *ANANAS COMOSUS* (L.) MERR.[1]

C. P. SIDERIS AND H. Y. YOUNG

Introduction

Well-authenticated information on pineapple plant response to nitrogen constitutes a valuable criterion in commercial fertilizer practices because the amounts and time of their application are determined by the physical appearance of the plants, designated as plant status, as well as by chemical analyses of their tissues.

Besides nutrition, physical factors such as light, temperature, and moisture may also influence the appearance or status of plants as measured by the chlorophyll content, succulence, and texture of the leaves. Under greenhouse conditions light was approximately 30% lower and temperature from three to five degrees higher than in the open.

Data presented in a previous publication (26) showed that growth and intake of nutrient mineral elements by *A. comosus* grown in nutrient solutions under greenhouse conditions increased more with high than with low amounts of nitrogen. Data for the same plants on dry matter, chlorophyll, titrable acidity, ascorbic acid, and various carbohydrate fractions are presented below.

Methods

Cultural methods were presented in a previous publication (26). The morphological basis of leaf nomenclature, the technique for sectioning the various organs, and the preparation of the tissues for chemical analysis have also been reported (22).

A brief review of the system of leaf nomenclature, originally reported elsewhere (22), may familiarize the reader with the different groups of leaves

and the position of their sections. Leaves having completed growth at the time of planting are designated as senile (A), and those having attained only partial growth as old (B). The leaves which are formed after planting are grouped according to their chronological age and physical appearance at the time of plant harvesting as mature (C), active (D), or young (E). All leaves are cut to four or five sections which may differ in physical appearance and chemical composition. The basal section, which is composed of meristematic tissues in leaves not having attained complete development and lacking in chlorophyll, is designated as no. 1, and the immediately adjoining section, which is partly chlorophyllous and occupies the position between the non-chlorophyllous and chlorophyllous sections, as no. 2. Section nos. 3, 4, and 5 are chlorophyllous but differ in chemical composition, presumably because of differences in chronological age and rates in physiological functioning.

Synoptic expressions have been introduced for the often repeated, long appellations of the cultures and series. Thus the cultures with 140.0 mg. of nitrogen per liter of solution are designated as high-N and those with 2.8 mg. as low-N cultures.

Review of literature

The literature on carbohydrate and nitrogen relations is very extensive. It is mainly concerned with the effects of varying amounts of nitrogen on carbohydrate accumulations in plant tissues and the interdependence of vegetative growth or fructification of such carbohydrate and nitrogen relations.

NIGHTINGALE's studies (18) on the nitrate and carbohydrate reserves in relation to the nitrogen nutrition of pineapples indicated that: ''It is essential to maintain an adequate reserve of nitrate in the plant. If the concentration of nitrate is relatively low . . . its reduction is not so efficiently or freely effected as when it is higher. Sufficient carbohydrates must be available for oxidation as nitrate is reduced.'' In a later publication (19) he reported: ''In the pineapple plant a low reserve of nitrate was found adequate for greatest possible yield of fruit when carbohydrates were low, in contrast with the requirements of the plants of high nitrate reserves for maximum production when carbohydrates were high.'' WADLEIGH (29) observed in cotton plants that hemicellulose reserves had not been affected at all, while sugars and starch were more abundant in the tissues of the low than high nitrogen cultures, but the yield of bolls was 5.3 times greater in the latter than former cultures. The studies of RUSSELL and BISHOP (21) on the relationships of nitrogen and carbohydrates in barley showed that: ''Nitrate assimilation is normally most active in the early part of the plant's life, and carbohydrate synthesis continues much later but its total amount depends on the quantity of nitrate taken up so that the two processes, though quite distinct, are nevertheless closely related.'' MURNEEK (15) observed that flowering of apples is characterized by marked increase of all active forms of carbohydrates and nitrogen, while leaf and fruit development is

characterized by an accumulation of nitrogen in water insoluble form which is plentiful in the leaves early in the season; later the nitrogen distribution is reversed.   TRAUB (**28**) noted that apple twigs contained, during the most active growth period, higher amounts of amino nitrogen, but lower amounts of protein nitrogen, total sugars, and total carbohydrates.   DAS (**3**) found in sugar cane that increasing nitrogen supplies increased the size of the leaf, the rate of leaf and joint formation, rate of elongation, tillering, yield of cane, uptake of ash constituents, soluble nitrogenous fractions, succulence, and content of reducing sugars, but decreased the concentration of sucrose in the expressed juice.   MACY (**10**) expounded a theory called "the relationship between sufficiency of a nutrient and its percentage content in the plant" which claimed to show that maximal yields of barley and straw are obtained when a certain critical percentage of nitrogen in the tissues is attained.   MACY's theory may be challenged by BOYNTON and COMPTON's (**1**) statement that chemical analysis of leaves for nitrogen, potassium, or magnesium cannot take the place of careful observations on tree behavior, appearance, and development of visible leaf or fruit symptoms; but that such analysis coupled with these observations may make possible a positive diagnosis that neither alone would have permitted.   LOEHWING (**9**) observed in oats, corn, and wheat that high yield in young plants was associated with high carbohydrates and high organic nitrogen, and low yield, with low protein, low carbohydrate and high nitrate content.   According to GAUCH and EATON (**4**), carbohydrate accumulations in barley plants grown in solutions with low and high concentrations of chlorides and sulphates were found to increase in the cultures with high concentrations of Cl or SO_4 which had also retarded plant growth.   MCILVANIE (**11**) found that the total nitrogen content of the roots of *Agropyron spicatum* was depleted, in the vegetative stage, to 53% of that present in the formative stage and carbohydrates, 70% of that occurring at the normal minimum; while the greatest relative amounts of reducing sugars were associated with rapid vegetative growth of sucrose with differentiation, and reserve polysaccharides with the brief rest period prior to secondary growth.

Results

GENERAL APPEARANCE

The high-nitrogen plants were very vegetative.   The leaves were quite long, broad, green, and soft by comparison with the low-nitrogen plants. The latter were smaller, with narrower, shorter, and stiffer leaves and with some die-back symptoms in the terminal regions of the relatively old or mature leaves.   The death of such regions was presumably caused by nitrogen deficiency.

DRY MATTER

Dry matter values reported as mg. per gram of fresh tissue were generally higher in the chlorophyllous sections (nos. 3, 4, and 5) of the leaves

FIG. 1 Dry matter in different sections of *A comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

for the high-N than low-N cultures in both nitrate- and ammonium-nitrogen series (fig. 1). Similar values in the combined basal and transitional (nos. 1 and 2) sections of the old (B) leaves which contained some chlorophyll were greater for the high-N than low-N cultures. In the nitrate-N series the basal (no. 1) and transitional (no. 2) sections of the young (E) leaves contained more dry matter for the high-N than low-N cultures. Dry matter values of the stem in the nitrate-N series were greater in the apical and medial but not in the basal sections for the high-N than low-N cultures. Similar values in the ammonium-N series, however, were greater for the low-N than high-N cultures. The roots contained more dry matter for the low-N than high-N cultures in both series.

FIG. 2. Chlorophyll in different leaf sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

Additional data indicate that differences in the dry matter content of comparable sections between different cultures resulted mostly from differences in the amounts of organic acids and sugars (table II and figs. 2, 3, 4, and 5).

CHLOROPHYLLOUS PIGMENTS

The chlorophyll and carotenoid content of tissues was greater for the high-N than low-N cultures (figs. 2 and 3). Concentrations of both pigments increased progressively from the basal (no. 1) to the terminal (no. 5) sections of the leaves except in a few sections. In *A. comosus*, as in all other plants, chlorophyll, depending on nitrogen for the synthesis of its molecule and for the proteinaceous stroma and grana of the chloroplasts, increased with greater supplies of nitrogenous fertilizers. The amounts of carotenoids in the various leaf sections correlated with those of chlorophyll, although nitrogen is not a component of the molecules of carotene or xanthophyll.

FIG. 3. Carotenoids in different leaf sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

The occurrence in relatively definite proportions in the cell of chlorophyll and carotenoids which are functionally interrelated suggests that their genesis may be synchronous. However, carotenoid formation in the stem and roots of *Daucus carota* and possibly in other plants is presumably independent of chlorophyll.

ACIDITY

Titrable acidity, reported as citric acid (fig. 4), was greater in the leaves of the high-N than low-N cultures. Minor exceptions which cannot be explained were observed in the chlorophyllous sections (no. 3) of the old (B) leaves of the low-N cultures in both series, also in sections of other leaf groups and in the stem of the nitrate-N series.
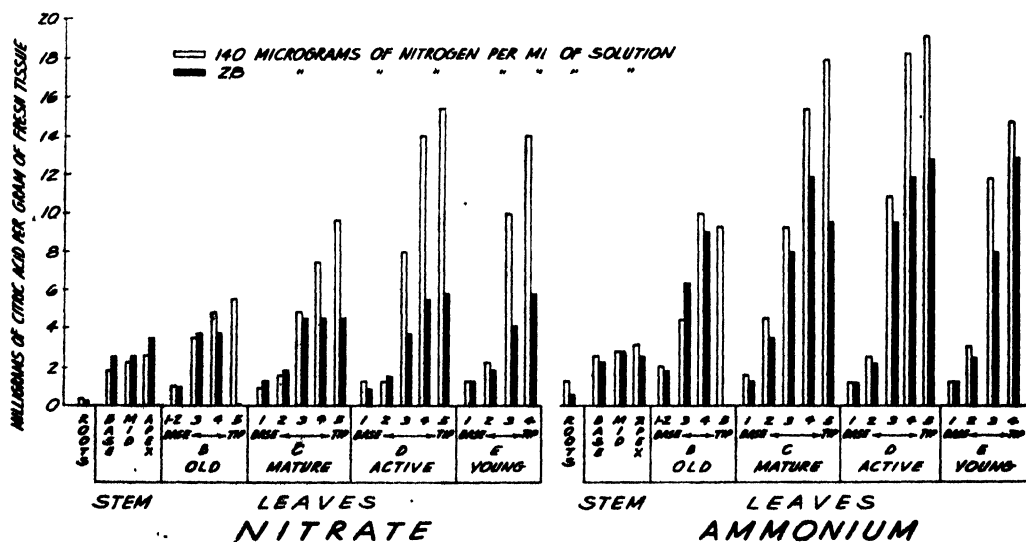
FIG. 4. Titrable acidity, as citric acid, in different sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

Preliminary studies (26) indicated that the organic acid content of chlorophyllous regions in leaves, as determined by weight, increased with greater plant vigor suggesting that the greater organic acid content of the high-N than low-N cultures had probably resulted from increased respiration caused by a higher rate of metabolic activity. Organic acids, presumably products of respiration, accumulated in plant tissues during the night or periods of darkness, possibly through enzymatic oxidation of sugars, and disappeared during the day or periods of light, also through enzymatic reduction to sugars or by further oxidation to carbon dioxide and water.

The acid content of non-chlorophyllous tissues in the basal leaf sections or in the stem or roots is not affected appreciably by diurnal changes, plant
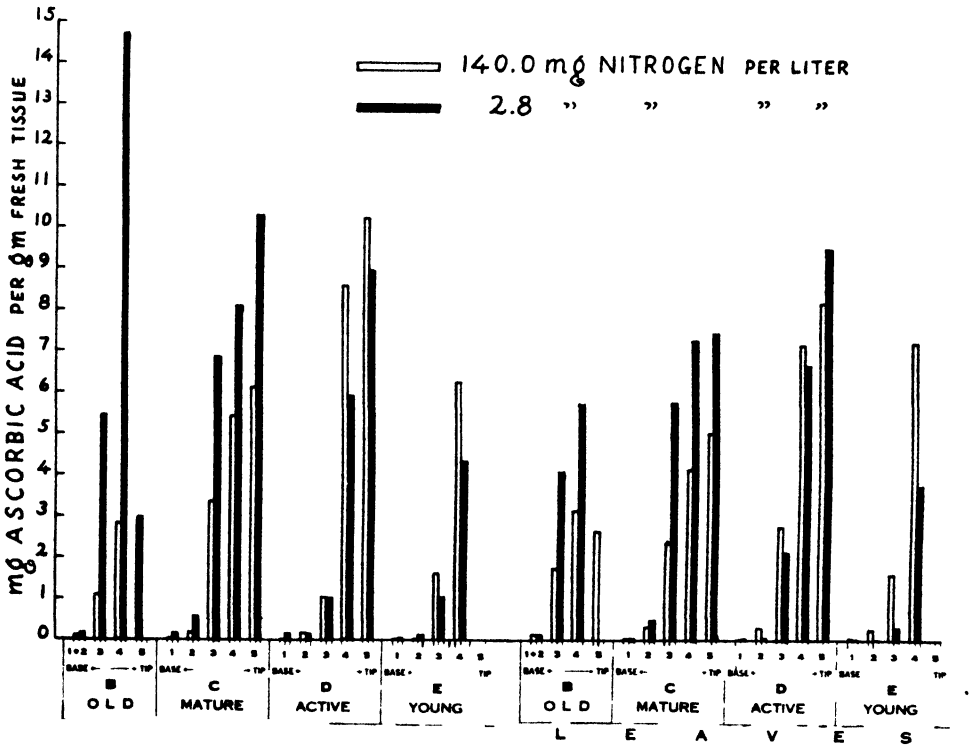
FIG. 5. Ascorbic acid in different leaf sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

vigor, or different kinds of nutrition. Therefore, in view of the accumulation of great amounts of organic acids in the chlorophyllous and exceedingly small amounts in the non-chlorophyllous sections, it would be misleading to associate both sections of the leaves with the same type of biochemical reactions generated during respiration. It is hypothetically possible that respiratory activity in non-chlorophyllous tissues lacking in ascorbic acid, a reducing agent, might involve the complete oxidation of sugars to carbon dioxide and water, whereas in the chlorophyllous tissues, amply supplied with ascorbic acid which is not affected by diurnal changes in the pineapple, sugars may be oxidized mostly to intermediary products, *e.g.*, malic, citric, and other carboxylic acids which tend to accumulate in the tissues.

Ascorbic Acid

The amounts of ascorbic acid in the chlorophyllous tissues of the leaves varied considerably between the high-N and low-N cultures (fig. 5). In the young (E) and active (D) leaves ascorbic acid was generally higher for the high-N than low-N cultures, but in the mature (C) and old (B) leaves the low-N contained more than the high-N cultures.

In view of the fact that our knowledge of the physiological functions of ascorbic acid in plant metabolism is limited, no satisfactory explanation can be offered for the greater accumulations of this substance in the young (E) and active (D) leaves of the high-N cultures or the converse in the low-N cultures.

Sugars

Total sugars showed greater accumulations for the high-N than low-N cultures (fig. 6). The gradients of sugar concentration in the tissues increased from the transitional (no. 2) to the terminal (no. 5) sections for the high-N but decreased for the low-N cultures in all except the young (E) leaf groups. In the latter group the sugar gradients increased from the transitional (no. 2) to the terminal (no. 5) section for the high-N and low-N cultures.

The chlorophyllous sections of leaves (nos. 2, 3, 4, and 5), associated with carbohydrate production by photosynthetic activity, and the basal sections (no. 1) composed of meristematic tissues and associated with carbohydrate utilization by formation of new tissues, present two contrasting aspects of the carbohydrate economy of the leaves. Ratios of mean sugar or of the combined sugar plus starch values of the chlorophyllous sections (nos. 2, 3, 4, and 5) to the basal section (no. 1) indicate the relative pressure or level of such carbohydrate substances in the producing (photosynthetic) and consuming (meristematic) regions (table I). The ratios show that a higher level was attained in the chlorophyllous than in the basal sections for the high-N cultures and the opposite or a negative pressure for the low-N cultures in all leaf groups except the young (E). The greater ratios of readily available carbohydrates (sugars and starch) in the chlorophyllous regions of the leaves of the high-N than low-N cultures should be attributed to the higher content of chlorophyll of the former cultures.

Starch

Starch concentrations in fresh plant tissues varied in the different cultures (fig. 7). In general the high-N cultures in the nitrate series contained more starch in the chlorophyllous sections of the leaves and in the stem than the low-N cultures. In the basal (no. 1) and transitional (no. 2) sections of the mature (C), active (D), and young (E) leaves with rapidly growing tissues, however, starch was more abundant for the low-N than high-N cultures. Similar values in the ammonium series were greater for the low-N than the high-N cultures. It was also indicated in former studies (24) that
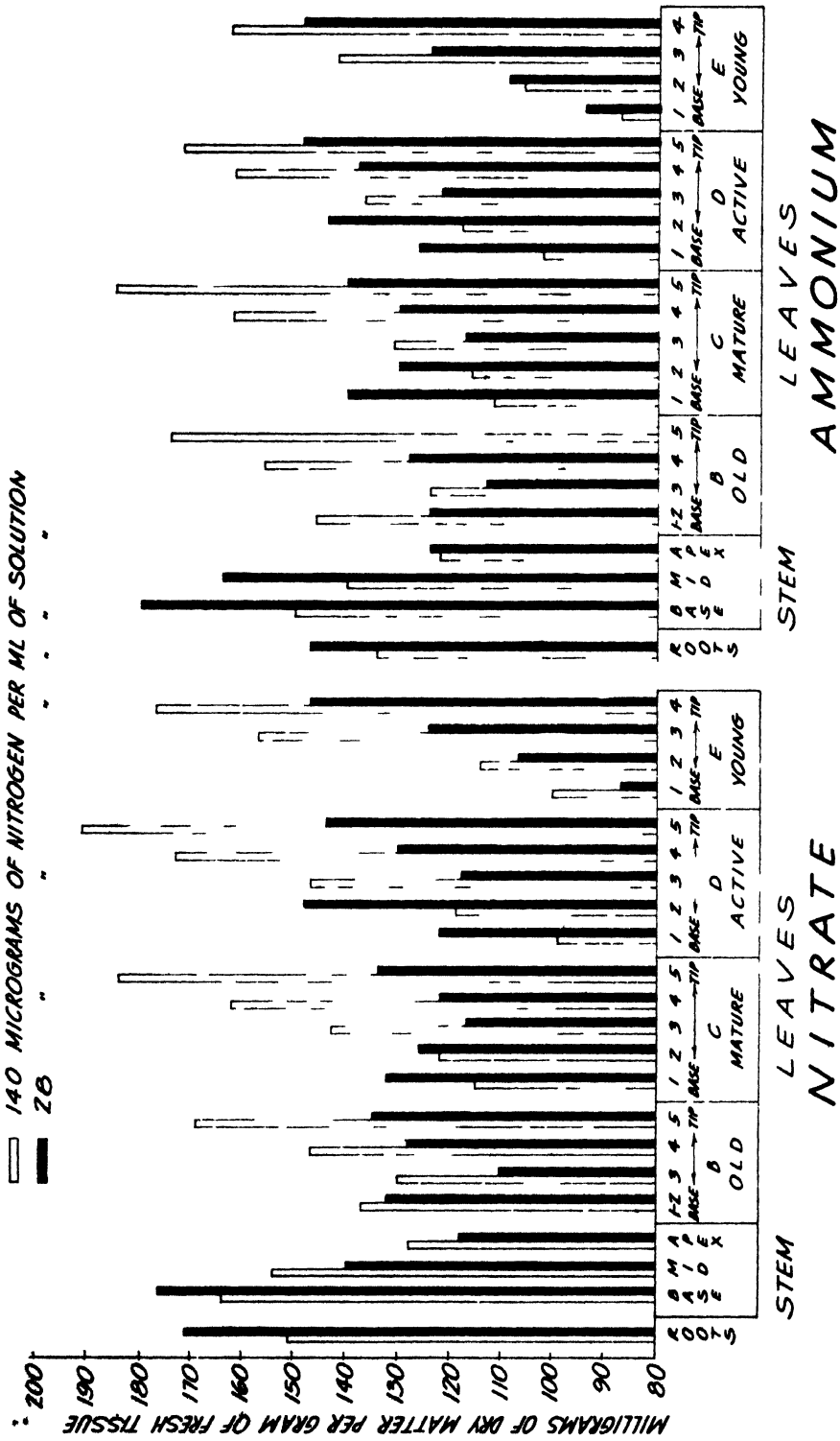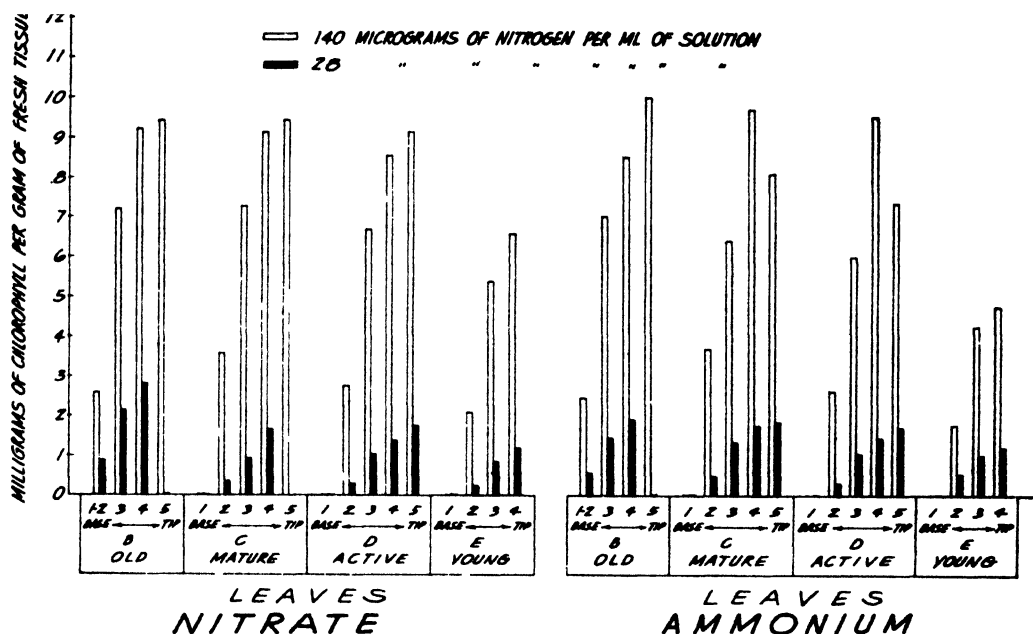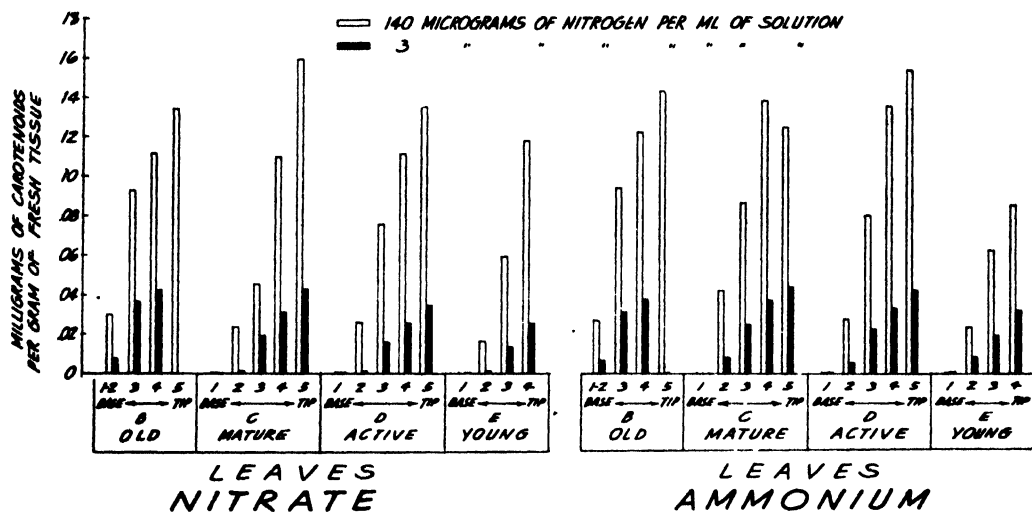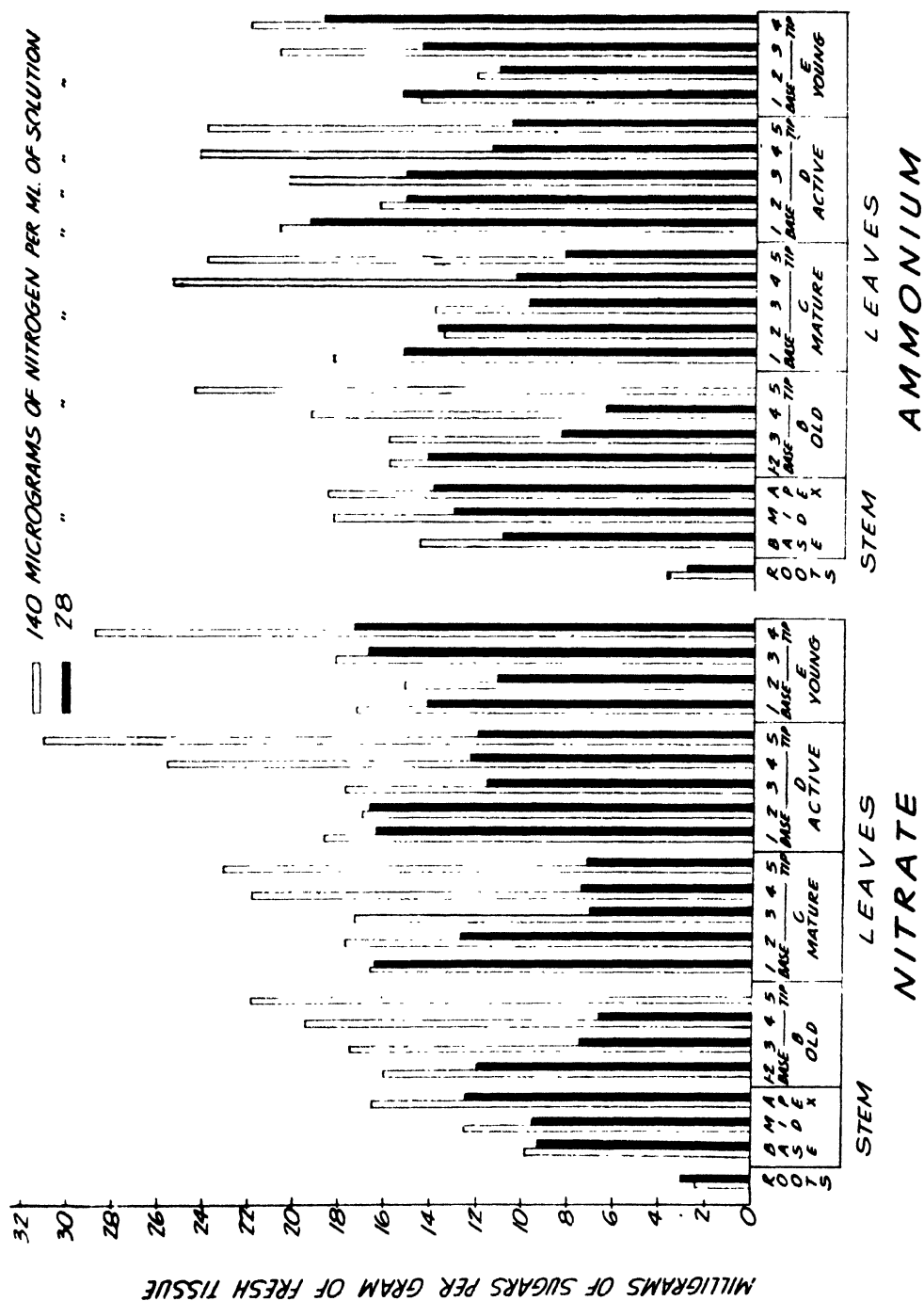
Fig. 6. Sugars in different sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

TABLE I

MEAN VALUES AND RATIOS OF TOTAL SUGARS OR TOTAL SUGARS PLUS STARCH FOR THE CHLOROPHYLLOUS (NOS. 2, 3, 4, AND 5) AND BASAL (NO. 1) SECTIONS OF DIFFERENT GROUPS OF LEAVES

| CARBOHYDRATE FRACTIONS IN LEAVES	NITRATE-N						AMMONIUM-N					
	HIGH-N			LOW-N			HIGH-N			LOW-N		
	CHLORO-PHYLLOUS SECTION	BASAL SECTION	RATIO OF CHLORO-PHYLLOUS TO BASAL	CHLORO-PHYLLOUS SECTION	BASAL SECTION	RATIO OF CHLORO-PHYLLOUS TO BASAL	CHLORO-PHYLLOUS SECTION	BASAL SECTION	RATIO OF CHLORO-PHYLLOUS TO BASAL	CHLORO-PHYLLOUS SECTION	BASAL SECTION	RATIO OF CHLORO-PHYLLOUS TO BASAL
	mg./gm.	mg./gm.		mg./gm.	mg./gm.		mg./gm.	mg./gm.		mg./gm.	mg./gm.	
Total sugars:												
Old (B)	19.50	16.00	1.22	5.00	12.00	0.42	19.50	15.80	1.23	5.60	14.10	0.40
Mature (C)	18.53	16.60	1.12	7.90	16.40	0.48	21.10	18.30	1.15	10.00	15.20	0.66
Active (D)	23.95	18.60	1.29	12.50	16.30	0.77	22.30	20.50	1.09	13.40	19.30	0.69
Young (E)	19.90	17.20	1.16	14.90	14.10	1.06	18.20	14.60	1.25	14.50	15.30	0.95
Starch:												
Old (B)	3.34	7.80	0.43	0.87	1.06	0.82	1.20	0.28	4.28	0.72	1.00	0.72
Mature (C)	3.82	2.26	1.69	2.39	4.86	0.49	1.22	0.15	8.13	2.80	9.72	0.29
Active (D)	4.33	2.20	1.97	6.27	8.23	0.76	0.51	0.28	1.82	3.72	13.70	0.27
Young (E)	4.45	1.48	3.00	1.90	0.41	4.63	0.20	0.28	0.71	1.06	0.62	1.70
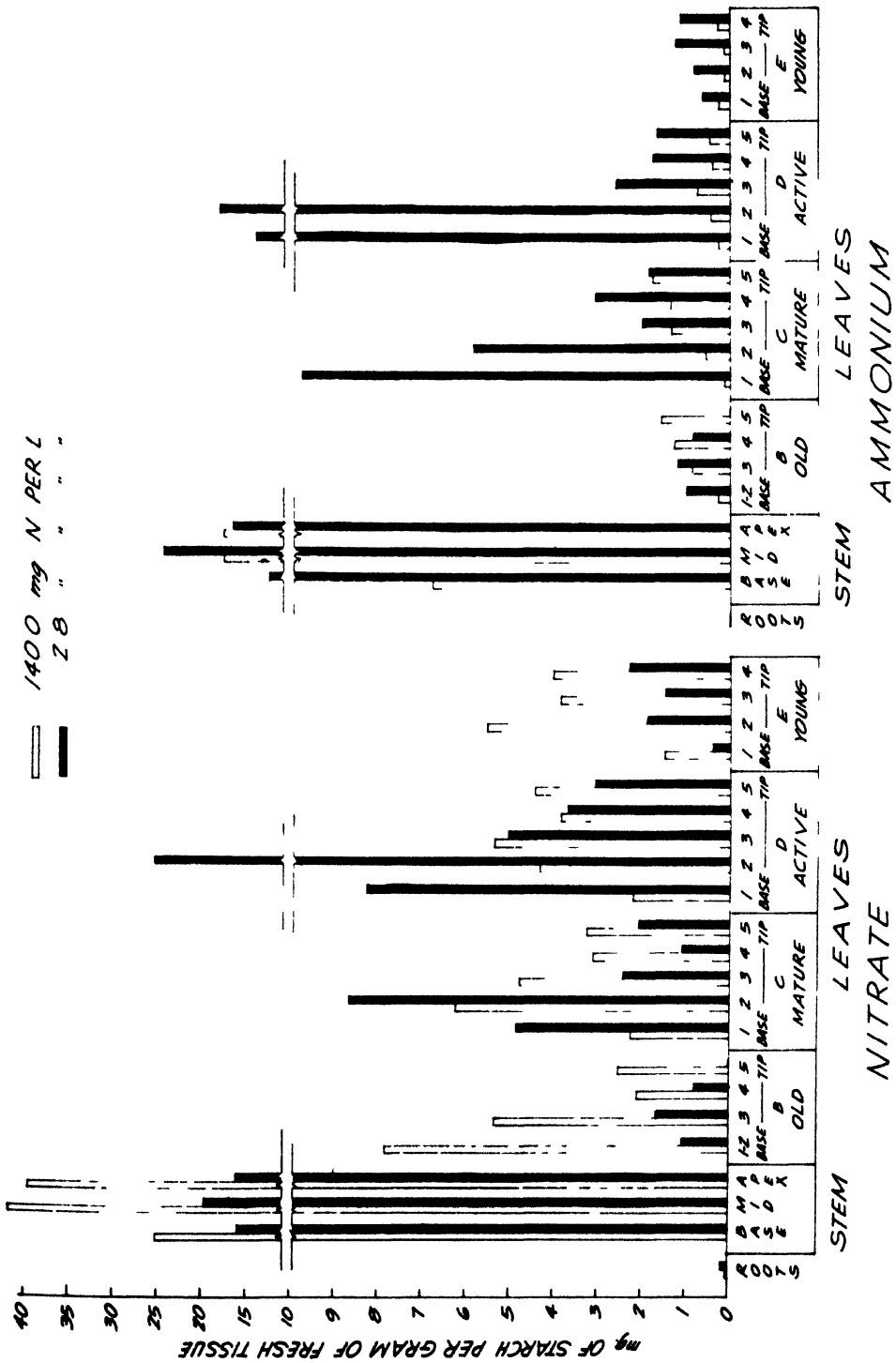
FIG. 7. Starch in different sections of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter supplied either as NO_3 or NH_4.

the lower starch values for the ammonium-N than nitrate-N series might have resulted from a high chloride content in the former series which interfered with optimum metabolic activities.   In this study starch ratios for the high-N cultures were greater in the chlorophyllous than in the non-chlorophyllous regions, *e.g.*, the meristematic tissues of the basal sections of the leaves, whereas similar ratios in the low-N cultures were smaller in the chlorophyllous than in the non-chlorophyllous basal sections except in the young (E) leaves (table I).

The data indicate that reduced growth activity caused by low supplies of nitrogen in the substratum may produce great starch accumulations in the basal (no. 1) and transitional (no. 2) sections which result from a low rate of carbohydrate utilization in the formation of new tissues.

<center>HEMICELLULOSE, CELLULOSE, AND LIGNIN</center>

Former studies (**23, 25**) indicated that no significant changes resulted from differential treatments of nutrition in the hemicellulose, cellulose, or lignin content of tissues, possibly because these substances representing structural and not energy-yielding units are relatively inert to the metabolic activities of the cell.   WINKLER and WILLIAMS (**30**), in *Vitis vinifera*, and WADLEIGH (**29**), in *Gossypium barbadense*, observed that the hemicelluloses are not affected by nutritional conditions because they are not utilized as sources of energy.

Total hemicellulose, and cellulose plus lignin values estimated on the basis of former findings (**24**) are compared with other carbohydrate fractions of the tissues (table II).

Discussion

The relation of nitrogen to carbohydrate synthesis and accumulation in plant tissues has been studied and discussed extensively by various investigators in association with vegetative growth and fructification under the title "carbon/nitrogen ratios."   THOMAS (**27**), reviewing the entire subject of carbon/nitrogen ratios, concluded as follows: (a) Carbohydrates increase in tissues with low growth activity and decrease with high activity; (b) nitrogen of the tissues is high before active growth, but it decreases with active growth, and (c) no specific carbon/nitrogen ratios have been found for any response.

Data herein presented cannot be evaluated in the light of THOMAS' conclusions with respect to fruit yields because they are concerned primarily with vegetative growth and not with fructification.   However, certain comparisons concerned with the vegetative growth of the high-N and low-N cultures and their content of total sugars and starch reveal the following: Sugar levels were lower in the basal (no. 1) sections with meristematic tissues than in the chlorophyllous sections of the leaves (nos. 2, 3, 4, and 5) for the high-N cultures with a high rate of vegetative growth and great carbohydrate utilization (fig. 8).   Similar levels for the low-N cultures with reduced vegetative growth were reversed, being higher in the basal (no. 1)

TABLE II

TOTAL AMOUNTS OF OBSERVED DRY MATTER AND RECOVERED SUBSTANCES PER PLANT (IN GRAMS AND IN PERCENTAGE OF OBSERVED DRY MATTER) IN ONE-YEAR-OLD *Ananas comosus* GROWN IN SOLUTION CULTURES WITH 140.0 OR 2.8 MILLIGRAMS OF NITROGEN PER LITER EITHER AS NITRATE OR AMMONIUM

DRY MATTER	NITRATE-N								AMMONIUM-N							
	HIGH-N				LOW-N				HIGH-N				LOW-N			
	PLANT	LEAVES	STEM	ROOTS	PLANT	LEAVES	STEM	ROOTS	PLANT	LEAVES	STEM	ROOTS	PLANT	LEAVES	STEM	ROOTS
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Cellulose + lignin*	139.00	118.00	7.85	21.00	87.00	55.40	4.04	34.30	106.63	92.20	5.43	9.00	107.50	76.80	5.26	28.25
Hemicellulose*	72.00	58.40	6.53	10.75	45.20	29.50	2.94	17.60	59.61	49.50	4.51	5.00	44.40	32.80	3.39	15.20
Sugar	65.45	60.11	4.57	0.77	23.26	20.31	1.53	1.42	58.27	53.30	4.34	0.63	28.13	24.84	2.12	1.17
Starch	25.50	11.52	13.97	0.01	8.53	5.95	2.57	0.01	5.28	1.74	3.53	0.01	8.74	5.66	3.05	0.03
Citric acid	23.62	22.64	0.88	0.10	7.02	6.50	0.42	0.10	30.30	29.36	0.74	0.20	16.63	15.95	0.44	0.24
Ascorbic acid	11.94	11.94	....	....	7.26	7.26	....	....	9.24	9.24	....	....	7.82	7.82	....	....
Chlorophyll P.	2.21	2.21	....	....	0.22	0.22	....	....	1.76	1.76	....	....	0.27	0.27	....	....
Org. N (×6.25)	54.10	45.53	5.24	3.33	11.20	7.88	1.00	2.32	61.00	55.80	3.67	1.53	11.62	8.72	1.08	1.82
Ash	44.34	37.12	5.03	2.19	31.65	26.87	2.19	2.59	29.23	25.73	2.65	0.85	35.50	31.26	2.40	1.84
Total recovered	438.16	367.47	44.07	38.15	221.34	159.89	14.69	58.34	361.32	318.63	24.87	17.22	265.61	204.12	17.74	48.55
Total observed	551.54	448.09	54.85	48.60	321.00	218.00	21.94	81.00	436.63	381.03	34.70	20.90	345.74	260.32	26.21	59.20
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Cellulose + lignin*	25.52	26.40	14.28	43.25	27.08	25.40	18.40	42.30	24.42	24.20	15.65	43.00	31.10	29.08	21.05	47.65
Hemicellulose*	13.04	13.00	11.90	22.15	14.68	13.55	13.40	21.70	13.65	13.00	13.00	23.90	14.30	12.60	12.95	25.50
Sugar	11.90	13.42	8.34	1.60	7.25	9.30	7.00	1.75	12.73	13.20	12.50	3.00	8.14	9.52	8.10	1.98
Starch	4.64	2.57	25.50	0.02	2.66	2.73	11.70	0.01	1.21	0.46	10.20	0.05	2.53	2.18	11.65	0.05
Citric acid	4.27	5.04	1.60	0.20	2.18	2.98	1.92	0.12	6.95	7.70	2.13	0.96	4.81	6.15	1.68	0.40
Ascorbic acid	2.17	2.64	....	....	2.26	3.33	....	....	2.12	2.42	....	....	2.26	3.01	....	....
Chlorophyll P.	0.40	0.49	....	....	0.07	0.10	....	....	0.40	0.46	....	....	0.08	0.10	....	....
Org. N (×6.25)	9.80	10.16	9.55	6.85	3.50	3.62	4.60	2.87	14.00	14.65	10.60	7.30	3.36	3.36	4.12	3.08
Ash	8.50	8.29	9.17	4.50	9.82	12.33	10.00	3.20	6.70	6.77	7.64	4.10	10.30	12.00	9.16	3.11
Total recovered	80.24	82.01	80.34	78.57	68.90	73.34	67.02	71.95	82.18	82.86	71.72	82.31	76.88	78.00	68.77	81.77

Estimated values on the basis of previous analyses (24).

than in the chlorophyllous (nos. 2, 3, 4, and 5) sections of the leaves, indicating a low rate of carbohydrate utilization and of synthesis. Differences in sugar levels between the basal (no. 1) and chlorophyllous (nos. 2, 3, 4, and 5) sections of the mature (C) and active (D) leaves were very small for the high-N cultures in the ammonium series, possibly because of interference with sugar utilization for the formation of new tissues by high chloride concentrations. Starch accumulated in greater amounts in the transitional (no. 2) and basal (no. 1) leaf sections of the low-N than high-N cultures, further corroborating the association of higher amounts of starch with plants of low vegetative growth. The relationship of higher amounts
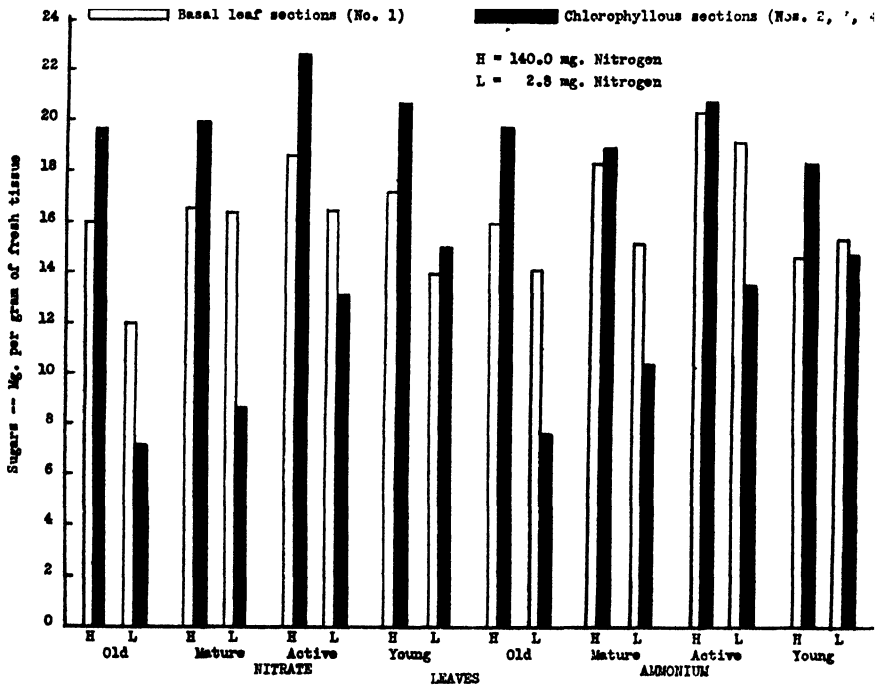
FIG. 8. Sugars, as mg. per gm. of fresh tissue, in the basal (meristematic) and chlorophyllous (photosynthetic) sections of the leaves of high-N and low N cultures.

of available carbohydrates (sugar and starch) with plants of low vegetative growth are in harmony with THOMAS' postulation *a*. (Postulation *b* will be discussed in a subsequent publication.)

Sugar and starch were higher in the basal (no. 1) and transitional (no. 2) than in the chlorophyllous (nos. 3, 4, and 5) leaf sections of the low-N cultures, whereas in the high-N cultures the levels were lower (figs. 6 and 7). These should be attributed to the restricted nitrogen supplies of the former cultures which caused reduction in the rate of growth and resulted in carbohydrate accumulations. Also, the higher chlorophyll content of the high-N cultures contributed toward greater carbohydrate synthesis. Results thus far indicate that physiological conditions which prompt reduction of vegetative growth also favor carbohydrate accumulations in plant tissues. Retardation of vegetative growth may result from insufficient amounts of all

essential mineral nutrients, water, or from traumatic and pathological factors; the latter might cause carbohydrate accumulations by interfering with the speed of carbohydrate translocation to other plant tissues or utilization for the formation of new tissues. Carbon dioxide, light, and temperature in insufficient amounts, although retarding vegetative growth, may cause carbohydrate depletion because they are directly associated with photosynthesis.

The relations of organic nitrogen fractions to carbohydrates have been studied by WOOD and PETRIE (31) who concluded: "there is no evidence that the steady state relations between proteins and amino acids may differ according to the carbohydrate concentrations."

Differences in various carbohydrates or other tissue contents between high-N and low-N cultures were greater for the former cultures when calculated on the basis of total plant weight than per gram of tissue because plant weights were greater for the former than latter cultures. For example, total sugars per plant were 181.5% and 107.0% greater in the high-N than low-N cultures for the nitrate-N and ammonium-N series, respectively (table II). Similar values per gram of fresh tissue (mean of all values) were 58.0% in favor of the high-N cultures for the nitrate-N and 45.8% for the ammonium-N series (fig. 6). However, the greater sugar concentrations (per gram of tissue) in the high-N than low-N cultures suggest that they resulted from a higher chlorophyll content in the former than latter cultures, but not from a reduced rate of vegetative growth.

Starch accumulations differed from sugars in relation to the chlorophyll content of the plants and rate of vegetative growth (table II). Between high-N and low-N cultures in the nitrate-N series starch differences per plant of fresh weight were 200.0% in favor of the former cultures, and in the ammonium-N series 65.5% in favor of the latter cultures; the lower starch content of the high-N cultures in this series resulted presumably from chloride toxicity. Similar differences per gram of fresh tissue (mean of all values) were 34.0% in favor of the high-N cultures in the nitrate-N series and 132.5% in favor of the low-N cultures in the ammonium-N series (fig. 7). Starch accumulations in the nitrate-N series were directly related to the chlorophyll content of the cultures, but in the ammonium-N series they were apparently influenced more by reduction in vegetative growth than by differences in the chlorophyll content of the tissues.

The data suggest that the small and insufficient nitrogen content of the low-N cultures decreased indirectly the sugar levels of the chlorophyllous sections by limiting chlorophyll synthesis within the available nitrogen supplies.

Preliminary studies (26) showed that titrable acidity in the chlorophyllous sections of leaves increases during the night or in darkness and decreases during the day or in light, and that such acidity associated presumably with metabolic activity correlated with plant vigor as measured by weight. Between high-N and low-N cultures acidity differences in the leaves

(in favor of the high-N cultures) were 85.3% for the nitrate-N and 29.3% for the ammonium-N series (fig. 4).

Ascorbic acid differences per organ (leaves) between high-N and low-N cultures were 64.5% and 18.2% in favor of the former cultures for the nitrate-N and ammonium-N cultures, respectively (table II). However, similar differences per gram of tissue (but in favor of the low-N cultures) were 43.0% for the nitrate-N and 20.6% for the ammonium-N cultures (fig. 5). Although total ascorbic acid per plant weight was greater in the high-N than low-N cultures, actual concentrations were lower in the former than latter cultures (table II and fig. 5). Such differences cannot be explained satisfactorily because of our limited knowledge of the physiological functions of ascorbic acid in plant metabolism.

Available information (5, 6, 7, 23, 24) indicates a significant parallelism between concentrations of ascorbic acid and of chlorophyll in different plants. However, MIRIMANOFF's (12, 13) and NEISH's (16, 17) studies present a different picture of the ascorbic acid and chlorophyll relationships; the former claiming a more probable association of ascorbic acid with flavo-

TABLE III

CHLOROPHYLL AND ASCORBIC ACID CONTENT AS MG. PER GM. OF FRESH TISSUE IN PLUS- AND MINUS-CHLOROPHYLL LEAF SECTIONS AND RATIO OF THE SAME OF (VARIEGATED) *A. bracteatus*

LEAF SECTIONS	CHLOROPHYLL	ASCORBIC ACID	ASCORBIC ACID/CHLOROPHYLL
	mg./gm.	*mg./gm.*	
Plus chlorophyll	0.501	1.20	2.40
Minus chlorophyll	0.064	0.54	8.43

nols than with chlorophyll, and the latter asserting that determination of ascorbic acid in separate chloroplasts did not show much difference between that and the leaves as a whole.

Determinations of ascorbic acid in adjoining leaf sections of variegated leaves of *Ananas bracteatus* show relations of ascorbic acid to chlorophyll for the plus- and minus-chlorophyll sections (table III).

Ratios of ascorbic acid to chlorophyll were greater for the minus- than for the plus-chlorophyll sections, suggesting that some other factor in addition to chlorophyll might have contributed toward ascorbic acid synthesis, or that its utilization under chlorotic conditions might not have been so great as under conditions of greater chlorophyll content in the tissues. Also, it is possible that ascorbic acid, being very soluble in the sap, may move by diffusion from the plus- to the minus-chlorophyll regions of the leaves, enriching the latter. Although there is some relationship between ascorbic acid and chlorophyll content of tissues, the amounts are not strictly proportional (23, 26).

GUHA and GHOSH (8) associate mannose as the raw material for ascorbic acid synthesis while BUKATSCH (2) and REID (20) claimed that sugars produced photosynthetically in leaves are responsible for increasing ascorbic acid content of the tissues of these organs.

Mean ascorbic acid values in the nitrate-N series were 3.63 mg. for the high-N and 5.26 mg. per gram for the low-N cultures, and in the ammonium-N series, they were 3.35 mg. for the high-N and 4.10 mg. for the low-N cultures. Mean chlorophyll values in the nitrate-N series were 0.622 mg. and 0.123 mg. per gram for the high-N and low-N cultures, respectively, while in the ammonium-N series they were 0.614 mg. for the high-N and 0.118 mg. for the low-N cultures. Ratios of ascorbic acid to chlorophyll in the nitrate-N series were 5.84 and 42.80 for the high-N and low-N cultures, respectively, and in the ammonium-N series, 4.47 for the high-N and 34.75 for the low-N cultures. Hence, the ratios of ascorbic acid to chlorophyll, indicating greater accumulations for the low-N than high-N cultures, may suggest some association of nitrogen with ascorbic acid in the metabolism of various nitrogenous compounds. MITCHELL (14) reported that v. Euler, Karrer, and Zehender found ascorbic acid or dehydroascorbic acid capable of dehydrating leucine with the formation of ammonia and strongly reducing volatile substances, and Abderhalden observed that in the presence of iron and oxygen, ascorbic acid deaminized and decarboxylated many amino acids.

Summary

The carbohydrate economy of *A. comosus* was studied in relation to high and low levels of nitrogen (140.0 and 2.8 mg. per liter) supplied to nutrient solutions either as nitrate or ammonium ions with the following results:

1. The percentage of dry matter in the chlorophyllous regions of healthy leaves was greater for the high-N cultures than for the low-N cultures, but in the non-chlorophyllous (basal) and transitional regions, the reverse was true. Similar values in the stem were greater for the high-N cultures in the nitrate series except in the basal section, but lower for the same cultures in the ammonium series.

2. Chlorophyll concentrations in leaf tissues were considerably greater for the high-N than low-N cultures. Carotenoids were also greater for the high-N than low-N cultures.

3. Titrable acidity, reported as citric acid, which by respiratory activity in the chlorophyllous leaf tissues increased during the night and decreased during the day, was greater in the high-N than low-N cultures and correlated in general with plant weights.

4. Ascorbic acid, limited mostly to the chlorophyllous regions of the leaves, varied in amounts between young or active and old or mature leaves. The amounts of ascorbic acid in the young or active leaves were greater for high-N cultures; but in the old or mature leaves they were greater for the low-N cultures.

5. Sugars in the leaves and stem were generally greater for the high-N than for the low-N cultures. The gradients of sugar concentrations increased from the transitional to the terminal leaf sections for the high-N cultures, but decreased for the low-N cultures except in the very young leaves.

6. Starch of the chlorophyllous sections of the leaves and of the stem, in the nitrate series, was greater for the high-N than low-N cultures, but in the non-chlorophyllous and transitional sections of the leaves, it was greater for the low-N cultures.   Nearly all leaf and stem sections in the ammonium series contained more starch for the low-N than high-N cultures.

7. The relations of nitrogen to vegetative growth, sugar, starch, and organic acid levels,in the tissues of plants were discussed in some detail.

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THE VITAMIN CONTENT OF SOYBEANS AND SOYBEAN SPROUTS AS A FUNCTION OF GERMINATION TIME

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(WITH THREE FIGURES)

The investigation upon which this report is based was made for the purpose of comparing the vitamin content of mature unsprouted soybeans of the Bansei variety with that of the same variety of soybeans after different periods of sprouting under controlled conditions, by a method suitable for yielding an edible product. Since the usual practice in oriental countries is to use the hypocotyls and to discard the cotyledons, the relative distribution of vitamins was ascertained separately in these portions of the sprouted soybeans after 54 hours of germination—the optimum period from the point of view of quantity coupled with organoleptic properties. The study included analyses of carotene, thiamin, riboflavin, niacin, and ascorbic acid content.

Sprouting was carried out under controlled conditions, following a technique found during preliminary trials to be favorable for edibility.

Historical review

Numerous studies have been made during the past three decades on one or more aspects of the nutritive value of sprouted soybeans and other legumes or grains; few studies, however, have dealt with products which have been prepared under conditions conducive to a high degree of edibility, and these have not been concerned, except in a few cases, with the relative merits of the hypocotyls and the cotyledons.

Early investigations in this field were confined chiefly to ascorbic acid content, using experimental animals for assessment purposes. Thus, in 1915, CHICK and DELF (12) demonstrated the antiscorbutic potency of germinated peas and lentils, and in 1917 WILTSHIRE (29) showed the same property in Haricot beans. EMBREY (15) in 1921 and SANTOS (26) in 1922 reported that the vitamin B content of Mung beans increased during germination.

From 1928 to 1940, MILLER and HAIR (23) studied the content of several vitamins in Mung beans, BOGARD and HUGHES (6) the ascorbic acid content of oats at different stages of sprouting, LEE and READ (19) the ascorbic acid content of sprouted soybeans in light and in darkness using the analytical method of BESSEY and KING (4), LEE (18) the ascorbic acid content of sprouted peas including the distribution of this vitamin through the cotyledons and the hypocotyls, ROSE and PHIPARD (25) the B vitamins in sprouted

peas and YEH (30) the thiamin content of unsprouted and sprouted Mung beans.

In 1942, BHAGVAT and NARASINGA RAO (5) showed an increase in ascorbic acid in sprouted Mung beans, reaching a maximum at 30 to 48 hours. Separate analyses were performed on the hypocotyls and cotyledons; the latter were found to be richer at the early stages of development.

In 1943, LUGG and WELLER (20), and BEESKOW (2) studied the ascorbic acid content of sprouted Mung beans and LANE (11) reported on the riboflavin content of black-eyed peas. Increases were reported after certain lapses of sprouting time in comparison with the fresh dry bean.

In papers reported from 1942 to 1945, Burkholder and associates described vitamin changes during the sprouting of beans and grains. Thus BURKHOLDER and McVEIGH (9) published values for thiamin, riboflavin, niacin, and biotin on dry and germinated Mung beans, lima beans, soybeans, peas, and wheat, although techniques of sprouting compatible with edibility were not followed. For soybeans germinated in liquid culture, there was usually a loss in vitamins during a preliminary soaking in a solution containing a low concentration of sodium hypochlorite. After four days of sprouting in a liquid culture the riboflavin had doubled and the niacin had increased one and one-half times in comparison with the initial dry bean; thiamin showed little increase.

BURKHOLDER (7) reported that grains—barley, corn, oats, and wheat—apparently synthesized certain of the vitamins during sprouting.

BURKHOLDER (8) gave the vitamin content of different varieties of soybeans. On the basis of micrograms per gram of dry matter, the Bansei variety contained 8.4 µg. of thiamin, 2.4 µg. of riboflavin, and 23 µg. of niacin per gram. The ascorbic acid value was found to be 0.21 milligram per gram of green raw beans. McVEIGH (21), in the same laboratory with BURKHOLDER, made studies on various parts of the oat seedling grown in the dark for five days, and reported marked increases in riboflavin and niacin, but none in thiamin, measured in content per seedling.

BURKHOLDER and McVEIGH (10) studied Canner King peas, the Mung bean commonly used in chop suey, and seven varieties of field soybeans. On a dry weight basis, riboflavin, niacin, and ascorbic acid increased greatly during germination, although thiamin showed little change.

FRENCH et al. (16) reported in 1944 on changes during sprouting in the vitamin content of peas, lima and kidney beans, as well as six varieties of soybeans. Some signs of germination were observed at the end of 24 hours, and, in most cases, sprouts were fully evident in 48 hours. The rates of germination and of vitamin formation were observed to be directly correlated with the temperature of the room.

Germination caused a marked increase in ascorbic acid, a fair increase in riboflavin and niacin, and none in thiamin. Average values for six varieties of soybeans after 72 hours of sprouting were 13.5, 0.29, 0.46, and 1.09 milligrams per 100 grams of the moist legume, for ascorbic acid, thiamin, riboflavin, and niacin, respectively.

Methods

One lot of Bansei soybeans was used throughout the study. In preparation for sprouting, the soybeans were washed four times with distilled water at 20° to 21° C., and then were hand-sorted. Broken fragments as well as beans with broken seed coats were discarded. The selected beans were soaked for 10 hours in a solution consisting of dilute calcium hypochlorite (5.5 grams per 10 liters of distilled water) to prevent mold growth. At the close of the soaking period, the soybeans were drained, washed with water at about 21° C., and placed in sprouting vessels which were kept in darkness in a constant temperature cabinet at 28° C. throughout the germination period. The conditions for sprouting were those found by preliminary trials to give a good edible product. Temperatures higher than those of the room were suggested by the work of EDWARDS (14), although his recommendation of 92° to 100°F. was not found to give so good a product as the temperatures used.

Enamel pans about 30 centimeters in diameter with handles on opposite sides served as sprouting vessels. The soybeans were placed on a double layer of cheesecloth suspended between the two handles. This provided the necessary drainage and permitted a layer of about three inches of water to be maintained below the cheesecloth to furnish water vapor. A piece of cheesecloth moistened with distilled water was placed over the beans, which were watered three times daily. Non-viable beans were removed as the sprouting progressed.

Two lots of soybeans were germinated, and analyses on duplicate samples were made in each case on the initial dry soybean, on the soybean after the 10-hour soaking period, and after 24, 48, 54, and 72 hours of germination under the conditions mentioned.

The method of WALL and KELLEY (28) as modified by COTTON (13) was used in the analysis of carotene. The modification includes immediate immersion and heating of food samples, the provision for the control of alcohol concentration in the lower phase during phasic separation according to AUSTIN and SHIPTON (1), and the recycling of the petroleum ether in the chromatographic separation. These changes permit better comparisons between the concentrations of carotene in raw and in processed fruits and vegetables.

Thiamin was determined by the Phycomyces method of HAMNER, STEWART, and MATRONE (17), riboflavin by the microbiological assay method of SNELL and STRONG (27), niacin by the procedure of MELNICK, OSER, and SIEGEL (22), and ascorbic acid (reduced and dehydro) by a combination of the methods of BESSEY (3) and of MORELL (24).

In making individual analyses of the dicotyledons and hypocotyls, the latter were separated carefully by hand. Moisture content was determined by drying in a 65° C. constant temperature oven for three days.

Results

The relative sizes of the initial dry and soaked soybeans, together with the comparative lengths of the sprouts after the different germination times are given (fig. 1). Figure 2 gives the number of soybeans in a 25-gram sample, initially, after soaking, and after the various germination periods;
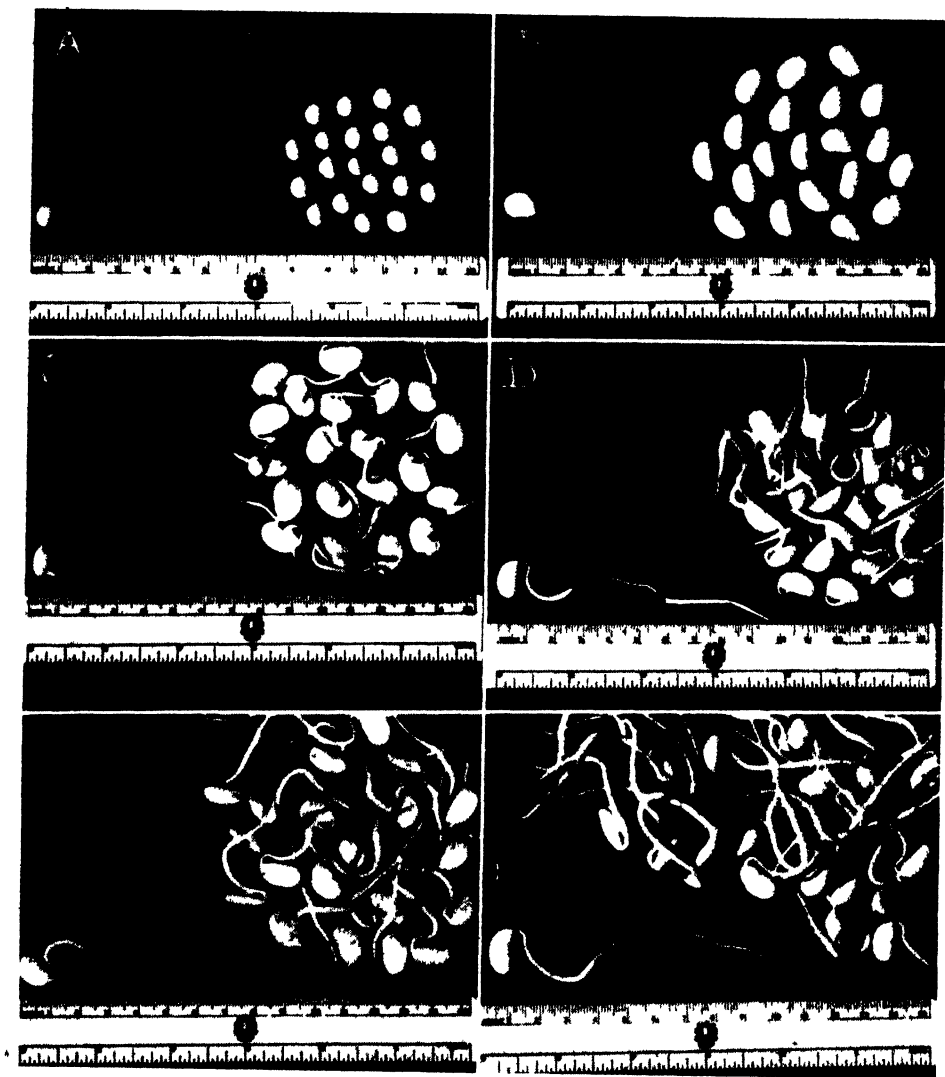
FIG. 1, A–F. Relative sizes of initial dry soybeans (A), of soybeans after soaking (B), and comparative lengths of sprouts after periods of germination of 24 hours (C), 48 hours (D), 54 hours (E), and 72 hours (F).

it gives, also, the vitamin content per soybean in the soaked bean, and in the sprouted bean after the four specified germination periods. Table I gives the vitamin content, calculated on the dry weight basis, of two sets of Bansei soybeans, after soaking and after four germination periods.

On an individual bean basis, there were but slight changes during the 10-hour soaking period in the content of those vitamins for which tests were

made, except for slight losses in thiamin and in reduced ascorbic acid, and slight gains in the dehydro form of ascorbic acid. The beans swelled considerably, however, and the number of soybeans per 25-gram sample decreased from 136 in the initial dry state to 56 of the soaked product in the first series, and from 137.5 to 55.5 in the second. The number of beans per 25-gram sample was reduced gradually, but only slightly during germination.

On the basis of average concentration per soybean, in the first series, carotene had almost doubled in 48 germination hours, and it had increased

TABLE I

VITAMIN CONTENT, DRY BASIS, OF TWO SETS OF BANSEI SOYBEANS AFTER SOAKING
AND AFTER FOUR GERMINATION PERIODS

HOURS OF GERMINATION	CAROTENE	THIAMIN	RIBO-FLAVIN	NIACIN	ASCORBIC ACID	
					REDUCED	TOTAL
	μg./gm.	μg./gm.	μg./gm.	μg./gm.	μg./gm.	μg./gm.
First series						
0*	1.2	12.8	2.1	27.6	8.7	88.3
24	1.2	17.2	2.8	34.1	198.0	273.6
48	2.2	9.0	3.0	50.8	429.0	517.0
54	3.8	8.4	4.5	45.8	469.4	627.5
72	4.3	11.0	5.6	45.8	354.9	626.3
Second series						
0*	1.3	14.0	2.1	21.0	0.0	108.1
24	0.8	19.7	1.8	21.6	141.6	199.8
48	1.9	9.7	2.7	33.6	421.6	582.3
54	1.6	9.5	4.0	30.0	488.6	599.7
72	2.0	13.4	5.0	50.5	557.9	705.2

* Soybeans after 10 hours of soaking in distilled water containing 5.5 grams of calcium hypochlorite in 10 liters of solution preparatory to soaking.

2.8-fold in 54 and 3.4-fold in 72 hours. In the second series, carotene increases were much lower; the maximum concentration was reached at 48 hours, with a 1.4-fold increase in 48 hours and no further change through 72 hours. The same trends were shown when the carotene concentrations were calculated on a dry weight basis.

Thiamin showed a slight gain in concentration during the first 24 hours of germination, followed by a loss through 54 hours, and a gain which brought the concentration close to the original between 54 and 72 hours. This trend was shown in both series, whether the calculation was made on an average per soybean or a dry matter basis. Further tests for this vitamin in other germination series showed that a periodic loss was followed by a gain in thiamin throughout the germination period of the soybean investigated.

Riboflavin showed a 1.9-fold increase in 54 hours of sprouting in the first series, calculated on an average per soybean basis, and a 2.5-fold increase in 72 hours (fig. 2). In the second series the increases were 1.8- and
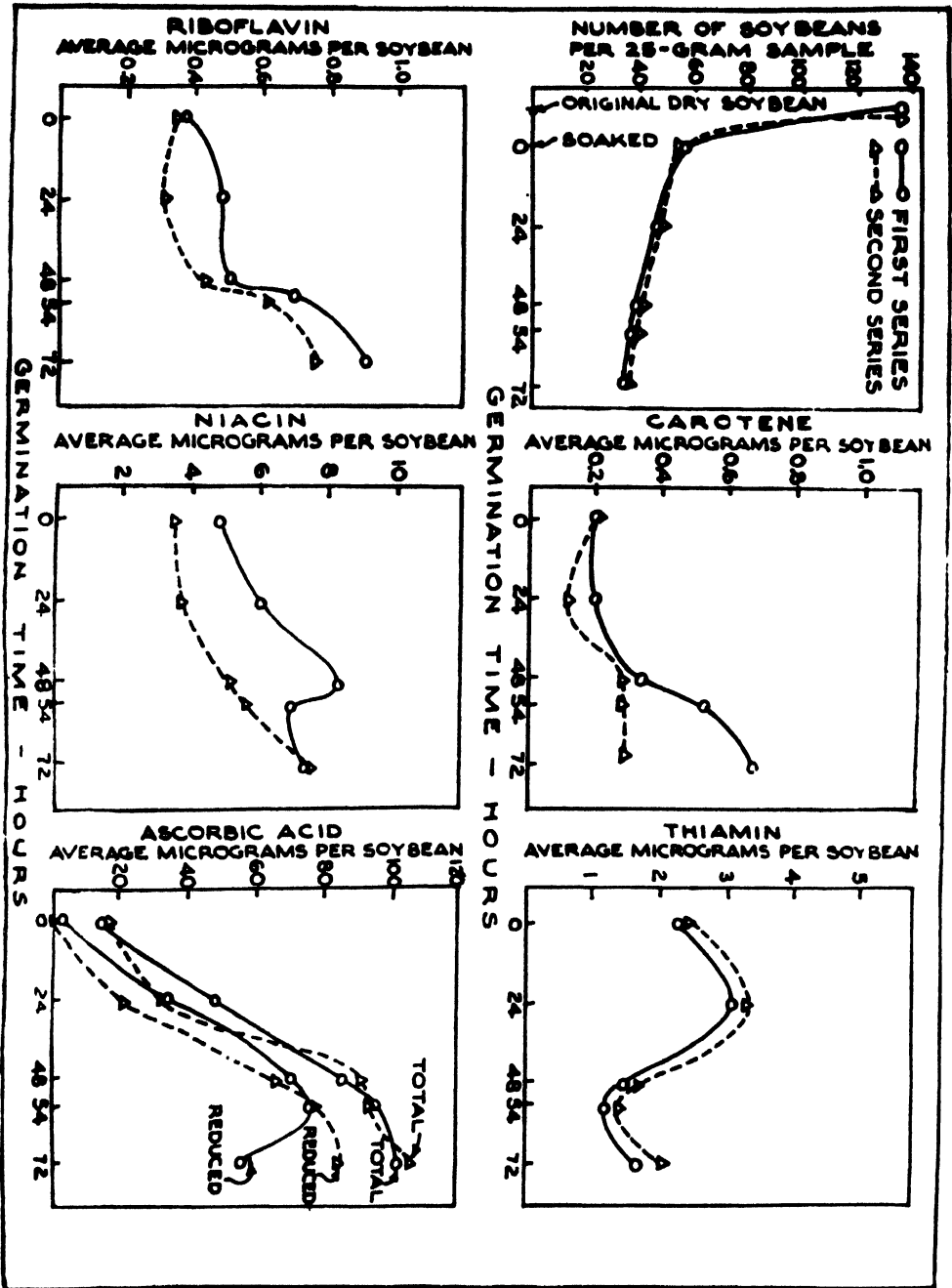
FIG. 2. Comparative number of soybeans in a 25-gram sample initially, after soaking, and after the respective germination times, average content per soybean (micrograms) of carotene, thiamin, riboflavin, niacin, and ascorbic acid (reduced and total) of the soaked bean, and of the bean after 24, 48, 54, and 72 hours of germination in the dark under conditions suitable for producing an edible product (28° C. in a cabinet providing temperature control).

2.1-fold for these respective germination periods. On a dry weight basis, the increases were 2.0- and 2.6-fold, and 1.9- and 2.4-fold for the two series and the 54-hour and 72-hour times of germination, respectively.

In both series of tests, the average niacin content per soybean was approximately doubled in 72 hours of sprouting. In the first series the rate of increase was greater for the intermediate times, with a minimum reached at 48 hours. In the second the increase was steady through the 72 hours of observations. Calculations on a dry weight basis showed the same general trends.

Ascorbic acid content showed the most marked increases during germination of any of the vitamins studied. In one series the soaked product contained an average of 8.7 micrograms of reduced and 88.3 of total (sum of reduced and dehydro) ascorbic acid per soybean; in the second series, there was no measurable amount of the reduced and 108.1 micrograms of

TABLE II

RELATIVE DISTRIBUTION OF DIFFERENT VITAMINS IN THE HYPOCOTYLS AND
COTYLEDONS OF SPROUTED SOYBEANS
(Average micrograms in the designated portion of one soybean
sprouted 54 hours)

VITAMIN	FIRST SERIES		SECOND SERIES	
	HYPOCOTYL	COTYLEDON	HYPOCOTYL	COTYLEDON
Carotene	0.13	0.44	0.05	0.19
Thiamin	0.28	0.99	0.31	1.16
Riboflavin	0.14	0.55	0.16	0.46
Niacin	1.27	5.77	1.43	4.27
Ascorbic acid—Reduced	15.65	59.56	15.57	60.23
Ascorbic acid—Total	20.80	74.91	26.11	66.91

the dehydro form, average per soybean, in the soaked product before germination. Increases in both forms were marked after 24 hours; and the total had increased 5.9- and 5.4-fold in 48 hours in the two series, 7.1- and 5.6-fold in 54 hours, and 7.1- and 6.5-fold in 72 hours, for the two respective series. The concentration of reduced ascorbic acid fell in one series between 54 and 72 hours, although the total showed a slight increase during this period.

From the point of view of appearance and edibility, the 54-hour germination sample was superior to any of the others.

The distribution of the vitamins between the hypocotyls and the cotyledons of the sprouted soybeans after 54 hours of germination, in terms of average micrograms per bean are shown (table II and fig. 3). The cotyledons had almost four times as much carotene, more than three times as much thiamin, almost four times as much riboflavin, approximately four times as much niacin, and approximately three times as much total ascorbic acid as the hypocotyls, on individual soybean basis. The practice therefore of discarding the bean and retaining only the sprout involves a considerable loss in the vitamin content of the sprouted bean.
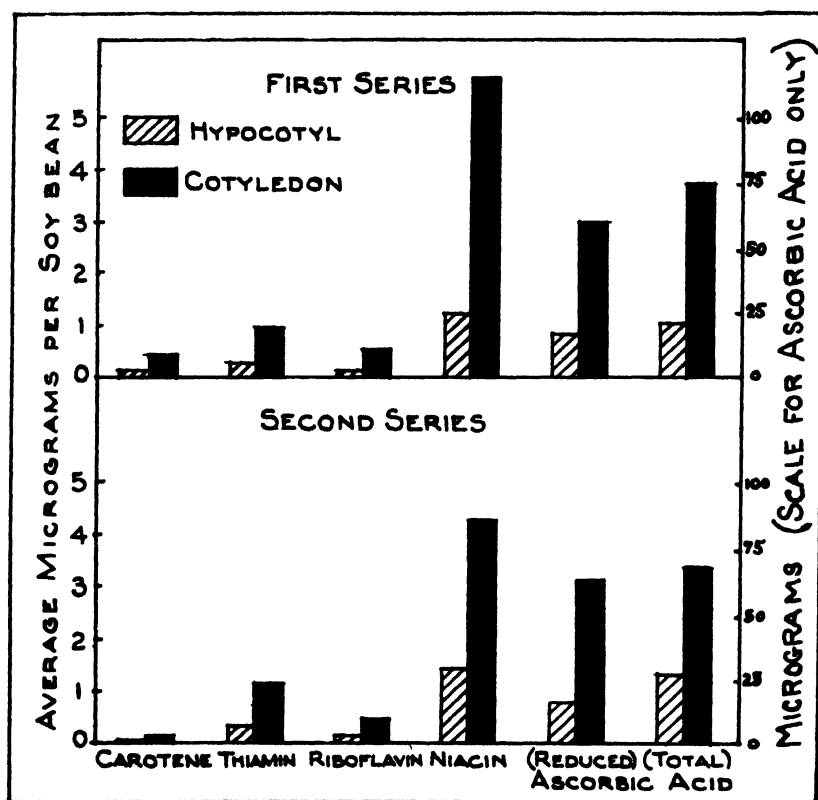
FIG. 3.   Distribution of vitamins in the hypocotyl and cotyledon portions of sprouted Bansei soybeans after 54 hours of germination.

Summary

Assays for carotene, thiamin, riboflavin, niacin, and reduced and dehydro ascorbic acid were made on dry Bansei soybeans, and on the soybeans after controlled soaking and germination periods; the latter were 24, 48, 54, and 72 hours.   The soaking and germination treatments were those selected after preliminary trials as giving a desirable edible product.   Analyses for the same vitamins were made on the hypocotyl and cotyledon portions after 54 hours of germination.

The quantity of all vitamins studied except thiamin showed increases through 54 hours of germination, the period at which the product exhibited its optimum organoleptic properties.   Thiamin showed alternate increases and decreases throughout the germination period investigated.

In the product of a 54-hour germination period, the cotyledons contained notably greater amounts of all vitamins for which tests were made.   The process of sprouting soybeans, therefore, increases the nutrient value of the product.   The entire bean should be eaten, since the cotyledons. which frequently are not retained, are a richer source of the vitamins for which tests are made than are the hypocotyls.

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EFFECTS OF NITROGEN ON THE NITROGENOUS FRACTIONS OF *ANANAS COMOSUS* (L.) MERR.[1]

C. P. SIDERIS, H. Y. YOUNG AND H. H. Q. CHUN

(WITH SIX FIGURES)

Introduction

The scope of this study is to investigate the amounts of certain nitrogenous fractions in the tissues of *A. comosus* grown in solution cultures with high or low amounts of nitrogen supplied either as nitrate or ammonium salts; two previous papers also concerned with the same plants had as objectives the growth and content of certain nutrient elements (35), chlorophyll, and various carbohydrate fractions in the tissues (36).

Former studies (30, 31, 32) have indicated that the leaves of *A. comosus* grown in substrata containing ammonium were greener, broader and more succulent by comparison with those grown in nitrate. Also, the tissues of the former contained no nitrate but greater amounts of soluble organic-N than the latter. Such morphological and chemical differences serve as indexes of nitrogenous nutrition and may be utilized under field conditions for the adjustment of the nitrogenous requirements of plants.

CULTURAL AND CHEMICAL METHODS

The composition of the culture solutions, employed for the growth of the experimental plants for one year, was reported in an earlier paper (35). The system employed for the nomenclature of the leaves, the technique for the sectioning of the various organs, the preparation of tissues and chemical methods for their analysis were reported in previous publications (29, 30).

Results

TOTAL NITROGEN

The data in table I, reporting the amounts of total nitrogen or its fractions as grams or as percentage of total nitrogen per plant or organ, show that the leaves comprised in the high-N cultures 85.46% and 91.63% and in the low-N cultures 68.80% and 69.14% of total nitrogen in the whole plant for the nitrate-N and ammonium-N series, respectively. Percentage of leaf weight for the same cultures, in the same table, was lower than percentage of nitrogen in the high-N cultures, but in the low-N cultures leaf weight percentage was higher than nitrogen percentage, indicating that the amount of nitrogen per unit of tissue was greater in the high-N than low-N cultures.

[1] Published with the approval of the Director as Technical Paper no. 171 of the Pineapple Research Institute, University of Hawaii.

TABLE I

TOTAL TISSUE CONTENT OF NITRATE-N, SOLUBLE ORGANIC-N, PROTEIN-N OR TOTAL-N PER PLANT OR ORGAN AND WEIGHTS FOR THE LATTER OF ONE-YEAR-OLD *A. COMOSUS* GROWN IN SOLUTION CULTURES WITH 140.0 OR 2.8 MILLIGRAMS OF N PER LITER SUPPLIED EITHER AS NO_3^- OR NH_4^+.

Nitrogenous Fractions or Plant Weights	Plant Organs	Nitrate Series				Ammonium Series			
		High-N		Low-N		High-N		Low-N	
		gm.	%	gm.	%	gm.	%	gm.	%
Nitrate	Leaves	0.449	5.26	......	......	......	......	......	......
,,	Stem	0.164	1.93	......	......	......	......	......	......
,,	Roots	0.058	0.68	......	......	......	......	......	......
,,	Plant	0.671	7.87	......	......	......	......	......	......
Soluble Organic-N	Leaves	1.515	17.80	0.245	14.30	3.049	31.00	0.217	11.34
,,	Stem	0.393	4.60	0.052	3.03	0.334	3.40	0.108	5.65
,,	Roots	0.116	1.34	0.035	2.04	0.047	0.50	0.038	1.98
,,	Plant	2.024	23.74	0.332	19.37	3.430	34.90	0.363	18.97
Protein-N	Leaves	5.313	62.40	0.935	54.50	5.958	60.63	1.107	57.80
,,	Stem	0.217	2.55	0.111	6.47	0.240	2.45	0.228	11.90
,,	Roots	0.293	3.44	0.337	19.66	0.198	2.02	0.217	11.33
,,	Plant	5.823	68.39	1.383	80.63	6.396	65.10	1.552	81.03
Total-N	Leaves	7.277	85.46	1.180	68.80	9.007	91.63	1.324	69.14
,,	Stem	0.774	9.08	0.163	9.50	0.574	5.85	0.336	17.55
,,	Roots	0.467	5.46	0.372	21.70	0.245	2.52	0.255	13.31
,,	Plant	8.518	100.00	1.715	100.00	9.826	100.00	1.915	100.00
Weights (gram)	Leaves	2952	81.00	1755	74.00	2660	86.60	2035	78.00
,,	Stem	376	10.20	147	6.20	254	8.30	167	6.50
,,	Roots	322	8.80	473	19.80	156	5.10	403	15.50
,,	Plant	3650	100.00	2375	100.00	3070	100.00	2605	100.00

In the stem the ratio of nitrogen to total plant N was greater in the low-N than in the high-N cultures. Differences in nitrogen percentage of the stem between high-N and low-N cultures were greater in the ammonium-N than in the nitrate-N series, suggesting concentration effects, because stems as percentage of plant weights were also smaller in the latter cultures. In the roots nitrogen as percentage of total plant N was greater in the low-N than high-N cultures, and percentages of root weights were likewise greater in the low-N than high-N cultures, indicating positive correlation between amounts of nitrogen and root weights.

Total nitrogen values for the entire plant or leaves of the high-N cultures, reported as milligrams per gram of fresh tissue, were greater in the ammonium- than nitrate-N series (table II). Similar values for the low-N cultures were approximately the same in both series. Comparison of total nitrogen values between stem and leaves shows that they were greater for the former than latter organs in the low-N, but not in the high-N cultures. Total-N values for the roots were greater in the high-N than low-N cultures. The much greater total-N values in the ammonium-N than in the nitrate-N series indicate a greater rate of absorption of NH_4^+ than NO_3^-, which was also found in previous studies (30).

SOLUBLE NITROGEN

Soluble-N, comprising various compounds of inorganic-N and organic-N, was considerably greater for the high-N cultures in the ammonium than nitrate series (tables I, II; fig. 1). Differences in soluble-N between nitrate-N and ammonium-N series in the low-N cultures were very small excepting the stems. The leaves of the high-N cultures contained 17.80% and 31.00% of soluble-N for the NO_3 and NH_4 series, respectively (table I). But in the low-N cultures, similar values were 14.30% and 11.34% for the nitrate-N and ammonium-N series, respectively. Percentage of soluble-N in the stem was greater for the high-N than low-N cultures in the nitrate-N series, but in the ammonium-N series the percentage of soluble-N in the stem was greater for the low-N than the high-N cultures. In the roots percentage of soluble-N was higher in the low-N than high-N cultures.

Soluble-N gradients for the leaves of the cultures in the nitrate-N series had two maximal values, one in the basal (no. 1) section resulting from the sum of soluble organic-N and nitrate-N and another one in the terminal (nos. 4 or 5) sections from soluble organic-N alone (fig. 1). However, similar gradients for the high-N cultures in the ammonium-N series had only one maximal value in the terminal (nos. 4 or 5) sections, except in basal (no. 1) sections of the young (E) leaves, which resulted from soluble organic-N accumulations. But gradients for the low-N cultures in the ammonium-N series behaved irregularly, possibly because of the low nitrogen supply which limited accumulations of soluble organic-N.

NITRATE-N

Nitrate-N was found only in the high-N cultures of the nitrate-N series

TABLE II

MEAN VALUES OF NITRATE-N, SOLUBLE ORGANIC-N, PROTEIN-N AND TOTAL-N AS MILLIGRAMS PER GRAM OF FRESH TISSUE OF *A. comosus* GROWN IN SOLUTION CULTURES WITH 140.0 (HIGH-N) OR 2.8 (LOW-N) MILLIGRAMS OF NITROGEN PER LITER SUPPLIED EITHER AS NO_3^- OR NH_4^+

PLANT AND ORGANS	NITRATE SERIES							AMMONIUM SERIES					
	HIGH-N				LOW-N			HIGH-N			LOW-N		
	TOTAL N	NO_3^- N	SOLUBLE ORG-N	PROTEIN-N	TOTAL N	SOLUBLE ORG-N	PROTEIN-N	TOTAL N	SOLUBLE ORG-N	PROTEIN-N	TOTAL N	SOLUBLE ORG-N	PROTEIN-N
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Leaves	2.470	0.152	0.518	1.800	0.673	0.140	0.533	3.385	1.145	2.240	0.651	0.107	0.544
Stem	2.060	0.437	1.045	0.578	1.110	0.354	0.756	2.260	1.315	0.945	2.012	0.647	1.365
Roots	1.450	0.018	0.360	0.910	0.787	0.075	0.712	1.572	0.302	1.270	0.632	0.094	0.538
Plant	2.335	0.184	0.555	1.596	0.723	0.140	0.583	3.196	1.116	2.080	0.734	0.139	0.595

FIG. 1.   Milligrams of soluble-N per gram of fresh tissue in different plant sections of one year old *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of N per liter supplied as NO_3 or NH_4.

(fig. 2).   The low-N cultures in the same series lacked in nitrate because of the small external supply.   Of course the cultures in the ammonium-N series were completely devoid of nitrate, which is in agreement with previous findings (**30, 33, 34**).

Nitrate gradients generally decreased from the basal (no. 1) to the terminal (nos. 4 or 5) leaf sections because of rapid reduction and assimilation

FIG. 2.   Milligrams of nitrate-N per gram of fresh tissue in different plant sections of one-year-old *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of N per liter supplied as NO_3 or NH_4.

of NO_3 in the chlorophyllous tissues.   Similar gradients in the stem generally had maximal values in the medial and may have occasionally in the apical sections.. Unpublished data indicate that nitrate accumulations in the non-chlorophyllous tissues may attain, but seldom surpass, concentrations as high as 1.5 mg. per gram of fresh tissue, presumably because the rate of nitrate absorption is dependent on the amount of available utilizable energy at the absorbing regions of the root.   Such regions in *A. comosus*

FIG. 3.   Milligrams of amide-N per gram of fresh tissue in different plant sections of one-year old *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of N per liter supplied as NO_3 or NH_4.

beginning approximately 0.5 cm. from the root tip proper and extending from 1 to 10 cm. towards the base are characterized by whitish, succulent, non-lignified tissues with or without root hairs. Also, other unpublished data have indicated that nitrate concentrations in the non-chlorophyllous leaf regions may depend on the ratios of plant volume to the surface of absorbing root regions.

AMIDE-N

In agreement with previous studies (30), amide nitrogen in the tissues of the high-N cultures, was greater in the ammonium-N than nitrate-N series (fig. 3). In the low-N cultures only slight and insignificant differences in amide-N were observed between series. The basal sections of the young (E) leaves and the apical of the stem composed mostly of meristematic tissues contained considerable amounts of amide-N.

The results might suggest direct synthesis of amide-N from oxalacetic acid or α-ketoglutaric acid and ammonia in the ammonium series (5, 8) but in the nitrate-N series similar synthesis may be doubted. Some amide-N in the ammonium-N and probably all in the nitrate-N series may have resulted from protein breakdown. Lack of knowledge of any mechanism associated with the reduction and assimilation of nitrate renders any intelligent explanation of amide synthesis from nitrates impossible.

MONO-AMINO-N

Mono-amino-N for the high-N cultures was greater in the ammonium-N than nitrate-N series (fig. 4). The differences in mono-amino-N for the low-N cultures between series were small. The gradient of mono-amino-N in the basal sections of the young (E) leaves was reversed with respect to other leaves. The greater mono-amino-N content of the high-N cultures in the ammonium-N than nitrate-N series was presumably associated with a much greater intake by plants of NH_4^+ than NO_3^-, which is in agreement with previous findings (30). Mono-amino-N gradients in the leaves increased from the basal (no. 1) to the terminal (nos. 4 or 5) sections except in the young (E) leaves where the content of the basal (no. 1) was greater than of the transitional (no. 2) sections. Similar gradients in the stem increased from the basal to the apical sections.

BASIC-N

Basic-N in the tissues of the high-N cultures was greater in the ammonium-N than nitrate-N series (fig. 5). Similar values for the low-N cultures were slightly greater for some sections in the nitrate-N than ammonium-N series.

Gradients of basic-N in the leaves of the high-N cultures increased from the basal (no. 1) to the terminal (no. 5) sections and in the stem from the basal to the apical sections except the medial in the nitrate-N series. Basic-N levels in the high-N cultures being greater for the ammonium-N than

FIG. 4. Milligrams of mono-amino N per gram of fresh tissue in different plant sections of one-year-old *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of N per liter supplied as NO_3 or NH_4.
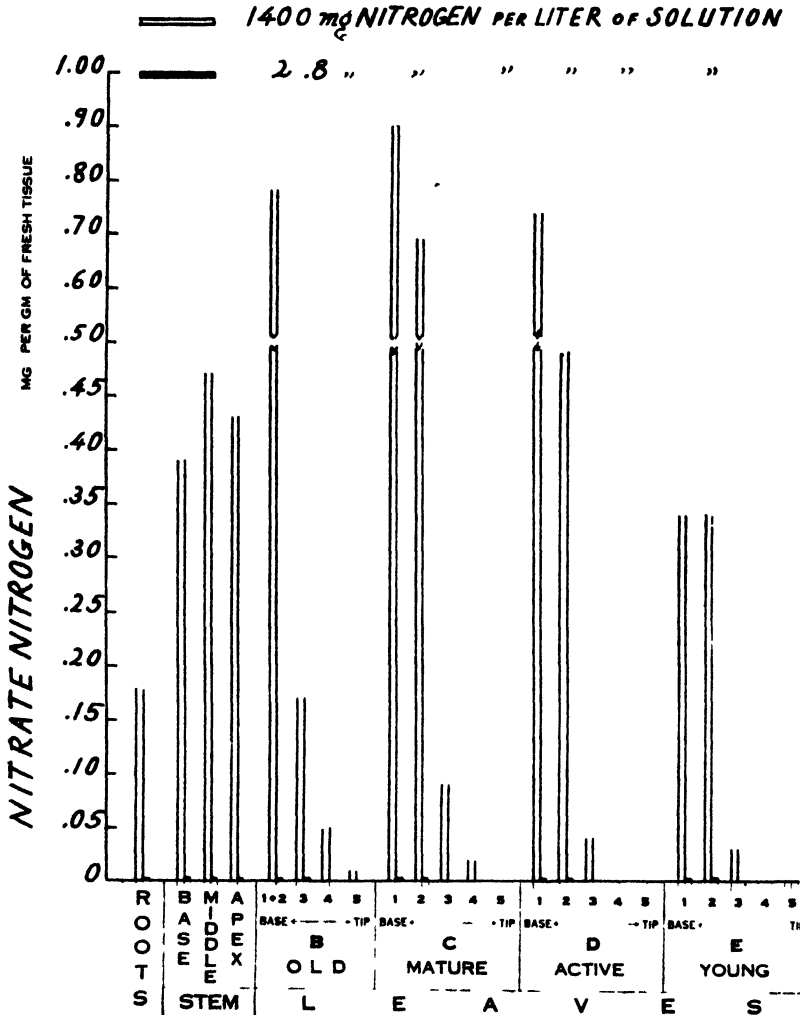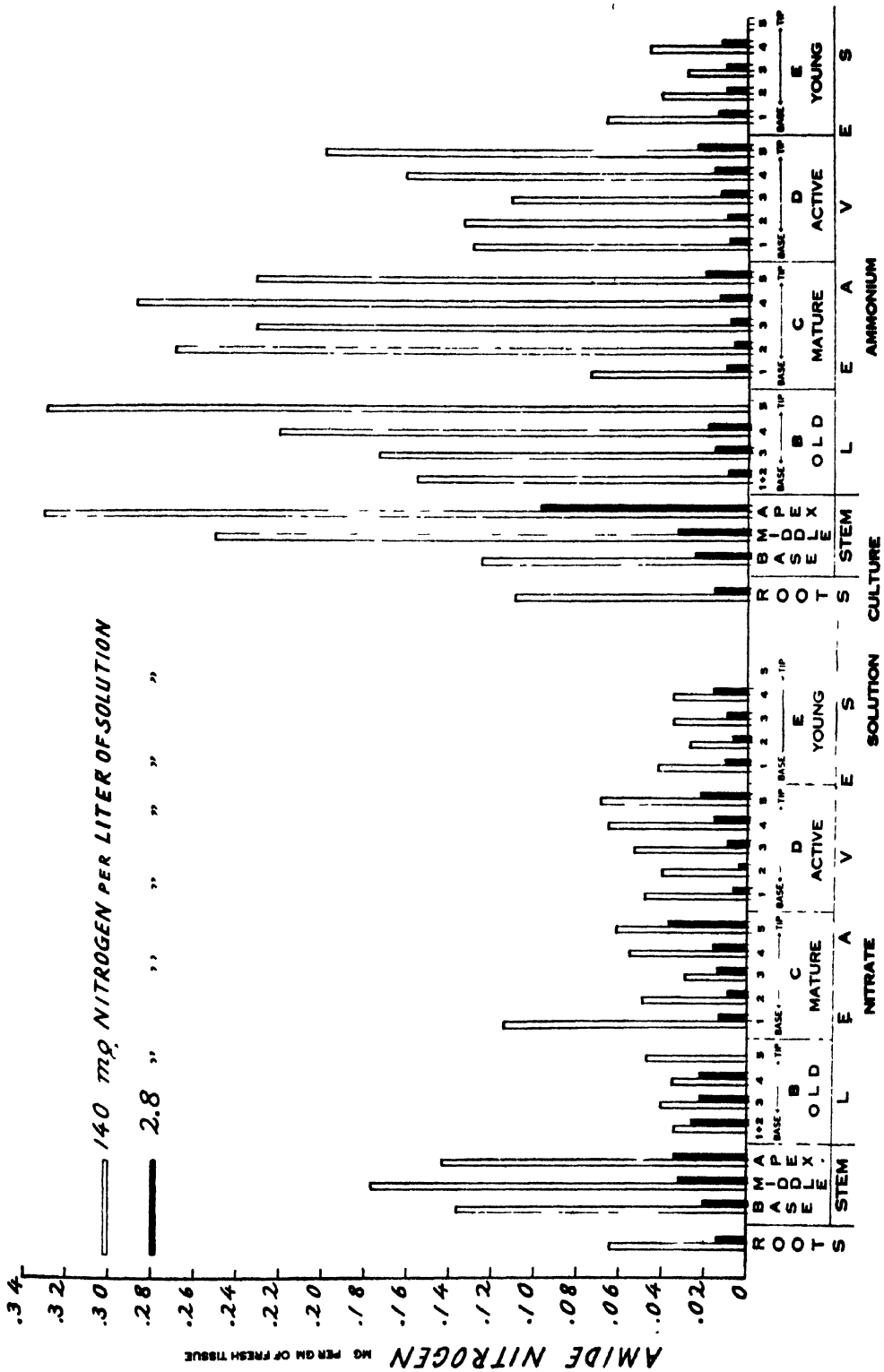
FIG. 5.   Milligrams of basic-N per gram of fresh tissue in different plant sections of one-year-old *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of N per liter supplied as NO_3 or NH_4.
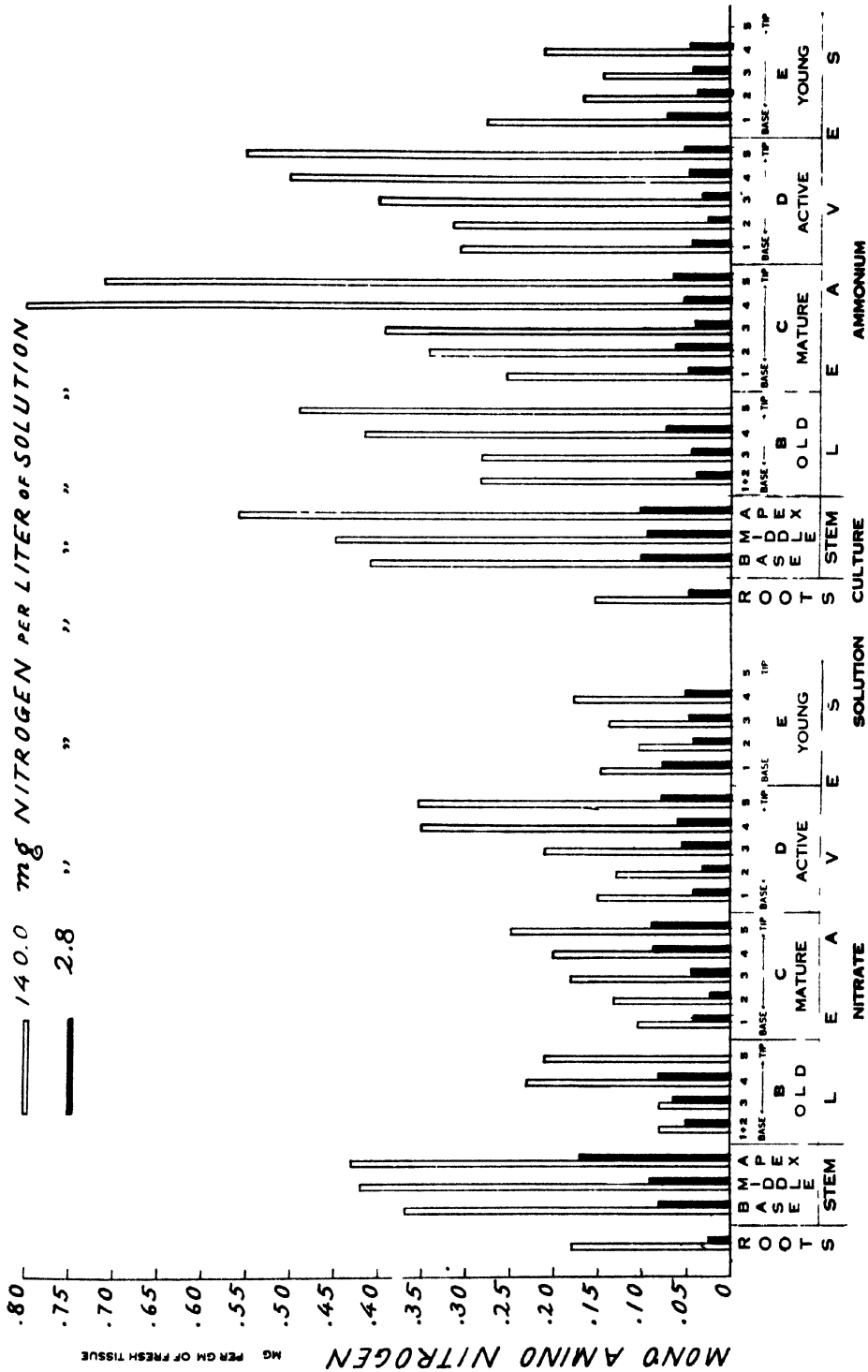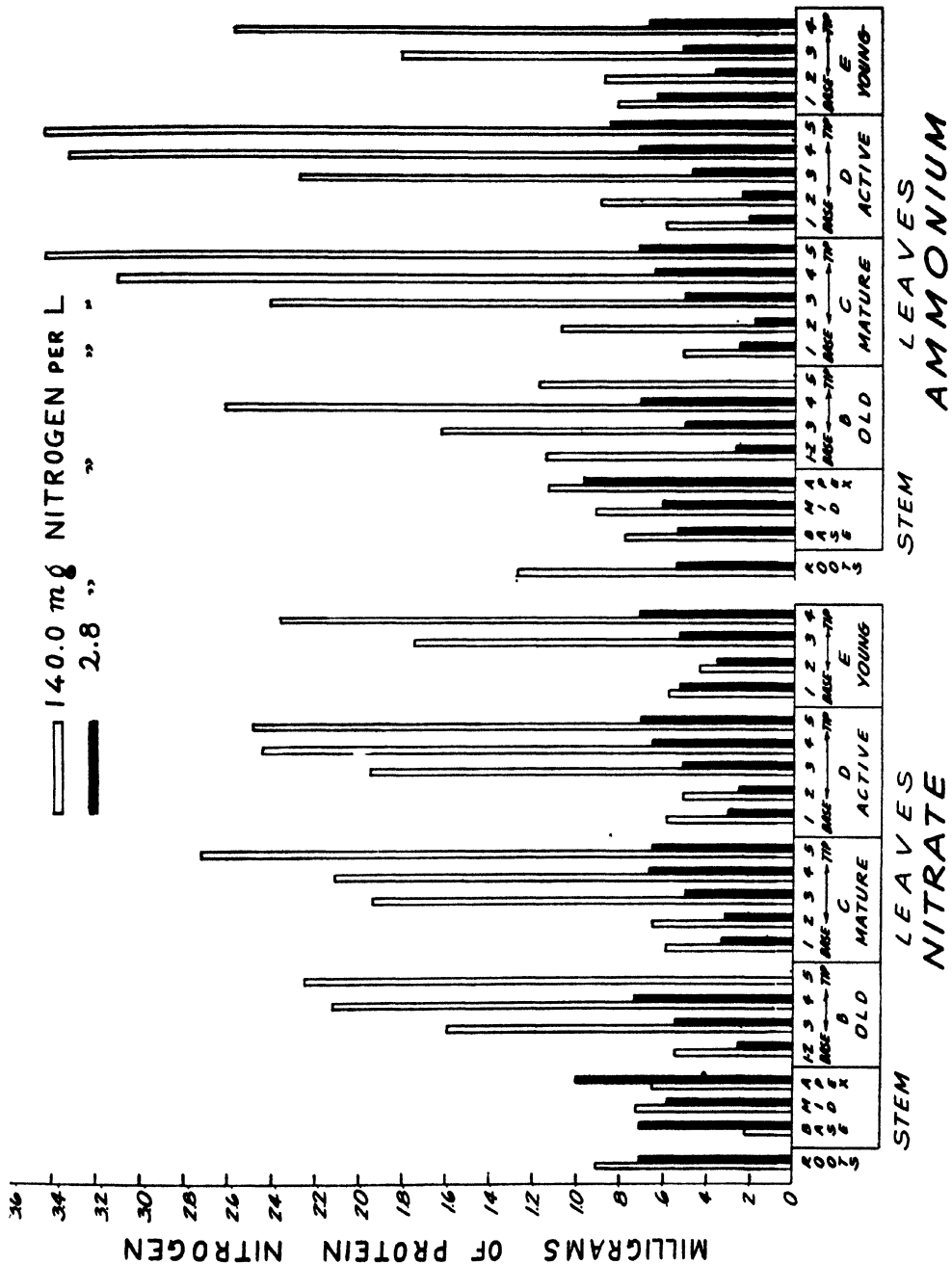
FIG. 6.   Milligrams of protein-N per gram of fresh tissue in different plant sections of one-year-old *A. comosus* grown solution cultures with 140.0 or 2.8 mg. of N per liter supplied as NO_3 or NH_4.

nitrate-N series cannot be attributed to direct synthesis from ammonium with some carboxylic acids and might have resulted from protein breakdown because experimental evidence on basic amino acid synthesis is lacking; but according to MOTHES (17), and KLEIN and TAÜBOCK (12), the content of free base in the sap may depend on the rate of reutilization for protein synthesis.

PROTEIN-N

Protein-N for the high-N cultures was greater in the ammonium than nitrate-N series (fig. 6). Similar values for the low-N cultures, however, showed slight differences between series, presumably because of limited external nitrogen supplies. Protein-N accumulated mostly in the chlorophyllous tissues and correlated with the amounts of chlorophyll; the non-chlorophyllous tissues of the leaves or stem containing smaller amounts than the chlorophyllous tissues.

Differences in the protein-N content of the high-N cultures between series, in favor of the ammonium-N series, were approximately 31.0% for the combined mature (C) and active (D) leaves and 18.0% for the young (E) leaves. Such differences indicate that protein-N in leaves may vary depending on the chlorophyll content of the tissues, amounts and kind of inorganic-N supplied to the roots and rate of absorption and assimilation of same. Protein-N gradients in the leaves of the high-N cultures increased from the basal (no. 1) in the ammonium-N series or from the transitional (no. 2) in the nitrate-N series to the terminal (no. 5) sections. In the stem gradients increasing from the basal to the apical sections were observed in the ammonium-N series.

Discussion

McKEE (16), NIGHTINGALE (20, 21), CHIBNALL (8), PETRIE (25) and WOOD (45) have reviewed the various phases of nitrogen metabolism in green plants. Studies by the authors (30, 33, 34) showed that NH_4^+ is assimilated by the root tissues of *A. comosus* almost as soon as it is absorbed and converted to amide-N, amino-N and protein-N, whereas NO_3^- is translocated from the roots to the non-chlorophyllous regions of the leaves in the original state and assimilated in the chlorophyllous regions. VICKERY *et al.* (40) employing N^{15} in ammonium salts as a tracer, and in other experiments (41) found that tobacco plants also assimilated NH_4^+ in a similar manner as *A. comosus*. BURSTRÖM (6, 7) presented evidence that stored nitrate in the leaves of wheat plants is assimilated only in light and that with increasing light intensity there is an increase in the assimilation of nitrate, apparent assimilation of CO_2 and formation of sugar; whereas, in darkness, nitrate accumulated within the leaves is not assimilated and sugar is oxidized to CO_2, which is quantitatively given off as respiration CO_2. The same author (6) suggested that a different mechanism operates for the assimilation of nitrates by roots. Also the experiments of BLACKMAN and TEMPLEMAN (2) suggested that light is necessary for nitrate assimilation. However, nitrate

assimilation in light according to NIGHTINGALE and SCHERMERHORN (19) may be characteristic of certain species of plants but in others nitrate reduction may be effected in darkness and in light mainly by the fine rootlets. Energy utilization, possibly derived from carbohydrates, is prerequisite for the absorption of nitrate and other anions from substrata, according to LUNDEGARDH (14), GILBERT and SHIVE (9), and HAMNER (11).

Experimental evidence for the conversion mechanism of NH_4^+ to amide-N and amino-N has been obtained (5) but not for NO_3^-. SCHULZE (28) expressed the opinion that amides arise by the reaction of ammonia, derived from amino acids of seed proteins, with nitrogen-free substances, while VICKERY et al. (42) believe that the precursors most probable for amino acids are the corresponding α-keto acids. CHIBNALL (8) suggested the citric acid cycle of KREBS and JOHNSON (13) providing a chemical mechanism for the formation of oxalacetic or α-ketoglutaric acids which may react with ammonia to form by enzymic activity aspartic, or glutamic acids or their respective amides, asparagine or glutamine. BRAUNSTEIN and KRITZMANN (5) demonstrated that amino nitrogen may be transferred to keto acids by transamination and ALBAUM and COHEN (1) showed that transamination between glutamic and oxalacetic acid occurs in oat seedlings. MOTHES (18) claimed amino acids may be formed enzymically in tissues either by combination of ammonia with carboxylic acids or by protein hydrolysis. BORSOOK and HUFFMAN (4) effected synthesis of aspartic acid from fumaric acid and ammonia, and of alanine from pyruvic acid and ammonia. VIRTANEN and LAINE (43) claimed that aspartic acid is the first amino acid formed in plants from which other amino acids may be produced by transference of the amino group to α-keto acids, but acid amides, e.g., glutamine or asparagine, cannot take part in transamination directly except after the splitting of the acid amide group. RAUTANEN (26), having studied transamination in green plants, claimed that in systems in which l-glutamic acid or α-ketoglutaric acid acted as the dicarboxylic acid component than l-aspartic acid or oxalacetic acid, transamination was quicker. Also, because of the formation of alanine and certain aliphatic amino acids through transamination in plants, l (–) aspartic acid pyruvic acid ⇌ Oxalacetic acid + l (+) alanine, he suggests that most amino acids can be regarded as derivatives of alanine.

Protein metabolism, in animals in nitrogen equilibrium, is, according to BORSOOK and KEIGHLEY (3), a process of continual breakdown of intracellular protein even when abundant quantities of amino acids are obtained from the diet, and as a consequence a corresponding quantity of amino acids is synthesized into tissue proteins and peptides. The literature pertaining to protein metabolism in plants indicates that a mechanism may be operating for the protein-N ⇌ soluble organic-N reaction somewhat similar to that in animals as postulated by BORSOOK and KEIGHLEY (3) or SCHOENHEIMER et al. (27).

The data in table III indicating ratios of protein-N to soluble organic-N for the high-N and low-N cultures in the nitrate-N series suggest that there

TABLE III

MEAN VALUES OF TOTAL ORGANIC-N, PROTEIN-N AND SOLUBLE ORGANIC-N AS MILLIGRAM WEIGHT OF ENTIRE SECTIONS AND RATIOS OF PROTEIN-N (P. N.) TO SOL-UBLE ORGANIC-N (S. O. N.) FOR THE COMBINED SECTIONS AS INDICATED OF *A. comosus* GROWN IN SOLUTION CULTURES WITH 140.0 (HIGH-N) OR 2.8 (LOW-N) MILLIGRAMS OF NITROGEN PER LITER SUPPLIED EITHER AS NO_3^- OR NH_4^+

PLANT SECTIONS	NITRATE-N								AMMONIUM-N							
	HIGH-N				LOW-N				HIGH-N				LOW N			
	TOTAL ORG-N	PRO-N	SOL ORG-N	P. N. / S.O.N.	TOTAL ORG N	PRO N	SOL ORG-N	P. N. / S.O.N.	TOTAL ORG-N	PRO-N	SOL ORG-N	P. N. / S.O.N.	TOTAL ORG-N	PRO-N	SOL ORG-N	P. N. / S.O.N.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*	
Leaves:																
Old (B) 1+2	78.0	22.2	55.8	0.395	13.5	8.1	5.4	1.50	74.1	44.3	29.8	1.49	16.3	12.5	3.8	3.29
3–5	684.0	562.0	122.0	4.600	104.5	81.6	22.9	3.57	727.0	400.5	326.5	1.23	156.8	127.1	29.7	4.28
Mature (C) 1+2	278.0	89.7	188.3	0.477	23.6	19.3	4.3	4.50	171.3	82.6	88.7	0.93	22.0	14.0	8.0	1.75
3–5	2294.0	1838.0	456.0	4.040	280.5	214.2	66.3	3.24	2650.0	1602.0	1048.0	1.53	288.0	242.0	46.0	5.26
Active (D) 1+2	345.6	110.1	235.5	0.418	44.3	34.0	10.3	3.30	285.0	144.5	140.5	1.03	45.8	33.5	12.3	2.72
3–5	2497.0	1924.0	573.0	3.355	382.3	307.5	74.8	4.10	3440.0	2401.0	1039.0	2.31	471.4	406.2	65.2	6.23
Young (E) 1+2	408.0	109.2	298.8	0.365	146.2	114.2	32.0	3.57	540.0	342.5	197.5	1.73	163.2	131.1	32.1	4.08
3–4	785.0	652.0	133.0	4.900	186.0	156.7	29.3	5.37	1042.0	859.8	182.2	4.72	175.4	151.6	23.8	6.37
Total leaves:																
Av. 1+2	1109.6	331.2	778.4	0.412	227.6	175.6	52.0	3.38	1070.4	613.9	456.5	1.35	247.3	191.1	56.2	3.40
3–5	6260.0	4976.0	1284.0	3.865	953.3	760.0	193.3	3.93	7859.0	5263.3	2595.7	2.03	1091.6	926.9	164.7	5.63
Stem:																
Base+Mid. +Apex	872.6	216.6	656.0	0.330	161.0	109.4	51.6	2.12	581.0	239.0	342.0	0.70	173.1	115.3	57.8	1.99
Roots	532.0	294.0	238.0	1.236	372.0	337.0	35.0	9.63	245.0	198.0	47.0	4.22	255.0	217.2	37.8	5.75

TABLE IV

PERCENTAGE OF PROTEIN-N AND SOLUBLE ORGANIC N AS TOTAL ORGANIC-N IN THE COMBINED SECTIONS, REPORTED IN TABLE III, OF ONE-YEAR-OLD *A. comosus* GROWN IN SOLUTION CULTURES WITH 140.0 (HIGH-N) OR 2.8 (LOW-N) MILLIGRAMS OF NITROGEN PER LITER SUPPLIED EITHER AS NO_3^- OR NH_4^+.

PLANT SECTIONS	NITRATE N HIGH-N PROTEIN N %	NITRATE N HIGH-N SOLUBLE ORG N %	NITRATE N LOW-N PROTEIN N %	NITRATE N LOW-N SOLUBLE ORG-N %	AMMONIUM N HIGH-N PROTEIN-N %	AMMONIUM N HIGH-N SOLUBLE ORG-N %	AMMONIUM N LOW-N PROTEIN-N %	AMMONIUM N LOW-N SOLUBLE ORG-N %
Leaves:								
Old (B) 1+2	28.5	71.5	60.0	40.0	59.8	40.2	76.7	23.3
3-5	82.0	18.0	78.0	22.0	55.0	45.0	81.0	19.0
Mature (C) 1+2	32.0	68.0	81.8	18.2	48.2	51.8	63.6	36.4
3-5	80.0	20.0	76.4	23.6	60.4	39.6	94.0	16.0
Active (D) 1+2	31.9	68.1	76.8	23.2	50.7	49.3	73.1	26.9
3-5	77.0	23.0	80.3	19.7	69.8	30.2	86.0	14.0
Young (E) 1+2	26.7	73.3	78.2	21.8	63.4	36.6	80.4	19.6
3-4	83.0	17.0	84.4	15.6	82.6	17.4	86.5	13.5
Total leaves:								
Average 1+2	29.0	71.0	77.3	22.7	57.4	42.6	77.3	22.7
3-5	79.5	20.5	79.8	20.2	67.0	33.0	85.0	15.0
Stem:								
Base + Mid. + Apex	24.9	75.1	68.0	32.0	41.2	58.8	66.4	33.6
Roots	55.3	44.7	90.5	9.5	80.8	19.2	85.2	14.8

is possibly a continuous breakdown of proteins to amino acids or conversion of the latter to the former. If we assume that the ultimate fate of nitrogen in plant metabolism is protein synthesis and that the reaction, soluble organic-N → protein-N is irreversible, then the presence of any soluble organic nitrogen fraction in substantial amounts in the tissues should constitute an anomaly except in cultures supplied with great amounts of inorganic nitrogen and operating under conditions retarding the conversion of soluble organic-N to protein-N. Even the tissues of the low-N cultures, grown under conditions of partial nitrogen starvation, contained appreciable amounts of soluble organic nitrogen (table III) which, calculated as percentage of total organic nitrogen (table IV), was almost as high as that of the high-N cultures. The ratios of protein-N to soluble organic-N in the

TABLE V

CORRELATION COEFFICIENTS (r) AND SIGNIFICANCE (t) AT 5% LEVELS OF PROTEIN N TO VARIOUS FRACTIONS OF SOLUBLE ORGANIC-N IN THE TISSUES OF DIFFERENT LEAF SECTIONS OF ONE-YEAR-OLD *Ananas comosus* GROWN IN SOLUTION CULTURES WITH 140.0 (HIGH-N) OR 2.8 (LOW-N) MILLIGRAMS OF NITROGEN PER LITER OF SOLUTION SUPPLIED AS NITRATE OR AMMONIUM SALTS

ITEMS OF CORRELATION	NITRATE SERIES				AMMONIUM SERIES			
	HIGH-N		LOW N		HIGH N		LOW-N	
	r	t	r	t	r	t	r	t
Soluble organic-N	− 0.78	*5.00*	0.59	*2.83*	0.66	*3.53*	0.52	*2.37*
Amide-N	0.31	1.30	0.43	1.85	0.33	1.40	0.46	2.00
Mono-amino-N	* 0.84	* *4.85*	* 0.56	* *1.90*	0.70	*3.92*	0.54	*2.47*
Basic-N	0.72	*4.17*	0.64	*3.23*	0.55	*2.62*	0.44	1.90
For statistical significance at:								
P .05		2.12		2.13		2.12		2.13
* P .05		* 2.26		* 2.31				

* Number of samples, 11, because of omission of basal sections; all other samples were 18.

chlorophyllous sections (nos. 3, 4, 5) of the high-N cultures were smaller in the ammonium-N than in the nitrate-N series because of much greater accumulations of soluble organic-N in the former series presumably resulting either from direct synthesis by combination of ammonia with carboxylic acids or from interference in the rate of conversion of soluble organic-N to protein-N by the toxic effects of high Cl^- concentrations (350 mg. per liter of nutrient solution).

In general protein-N as percentage of total organic-N in the proximal (nos. 1–2) sections was greater in the low-N than in the high-N cultures (table IV), presumably either because of a lower rate of protein hydrolysis in the former than latter cultures or because the rate of synthesis of soluble organic-N is dependent on the external supplies of inorganic nitrogen. Percentage of protein-N or soluble organic-N in the chlorophyllous sections

of the young (E) leaves varied slightly between different cultures because the tissues were chronologically young and insufficient time had elapsed for the accumulation of soluble organic-N.

The greater values of the ratios of protein-N to soluble organic-N.(table III), in the chlorophyllous sections (nos. 3, 4, 5) than in the non-chlorophyllous or partly chlorophyllous sections (no. 1, 2) should be attributed to the greater chlorophyll content of the former sections, which was found to correlate with the protein content of the tissues. Total nitrogen in the tissues increased in general with greater amounts of inorganic nitrogen in the nutrient solutions and *vice versa* (figs. 1–6).

Protein-N correlated directly with most fractions of soluble organic-N in the tissues (table V). The data indicate that correlation between protein-N and amide-N was lacking in all cultures. However, correlation between protein and mono-amino-N or basic-N was highly significant for the high-N cultures. Similar correlation was found in the low-N cultures except for mono-amino-N and basic-N in the nitrate-N and ammonium-N series, respectively. Moreover, in order to obtain definite correlation for mono-amino-N in the nitrate series it was necessary to exclude the non-chlorophyllous (no. 1) and partly chlorophyllous or transitional (no. 2) sections because the content of mono-amino-N in relation to protein-N was too high. It is assumed that a portion of this mono-amino-N was translocated to the proximal (nos. 1 and 2) sections of the leaves from the distal chlorophyllous (nos. 3, 4, 5) sections where it was derived by reduction and subsequent assimilation from nitrate-N. A similar condition did not obtain for the cultures in the ammonium series possibly because ammonium ions were assimilated by the root tissues as soon as they were absorbed and then translocated as fractions of soluble organic nitrogen. Therefore, correlation of protein-N to mono-amino-N and basic-N might indicate a continuous process of protein breakdown to mono-amino-N and basic-N and synthesis of the former by the reverse process.

WOOD and PETRIE (43) also found that definite relations existed between synthesis of protein in plant tissues and content of amino acids, ammonia and water. However, the rate of protein formation in tissues may be retarded by a decreased rate of synthesis of cystine, according to PETRIE and WOOD (23, 24) or of methionine, according to LUGG and WELLER (15). In order to explain the regulation of protein decomposition and synthesis in plants PAECH (22) formulated the "mass action" hypothesis which postulates that a single mass law reaction exists between proteins, on the one hand, and a carbohydrate and ammonia, on the other. A somewhat similar view is shared by GREGORY (10) who stated that if protein synthesis were the reverse of proteolysis, the direction of the reaction would be determined by mass action and the velocities of the reactions concerned. Variation in velocity of the reactions concerned would lead to accumulation; thus, if the synthetic route to protein were rapid, protein would tend to accumulate, and by mass action increase the breakdown to amino acid. If these amino

acids, before being again used in protein synthesis, were deaminated, and if this particular reaction were the slowest in the cycle, then amino acids would accumulate. However, the great complexity of factors already known (15, 25) to be associated with protein synthesis or breakdown weakens PAECH's hypothesis with respect to the effects of mass law action.

The direction and velocity of the reaction soluble organic-N $\rightleftharpoons$ protein-N for different plant sections (tables III and IV), which apparently were different for the chlorophyllous than non-chlorophyllous sections of the leaves, were probably affected by factors associated with the physiological functions of the tissues concerned in a somewhat similar manner as claimed by SCHOENHEIMER et al. (27) for animal tissues; the authors observed by means of N^{15}, employed as a tracer, that all the proteins of the body are continually undergoing synthesis and breakdown, the process being more rapid in some organs as the liver and kidneys than in others, as the erythrocytes. In the chlorophyllous sections (nos. 3–5) where considerable protein is a constituent of the chloroplastic stroma, protein-N values were generally greater than in sections 1 and 2, the former lacking in and the latter containing small amounts of chlorophyll. Protein amounts in the chlorophyllous sections did not vary as much for similar cultures between different series as did soluble organic-N. These results suggest that the amounts of soluble organic-N, attributable to protein breakdown, may be in the range of the ratios of protein-N to soluble organic-N, indicated in table III, for the low-N cultures because the greater soluble organic-N content of the high-N cultures might possibly contain small portions synthesized directly from ammonia and keto acids and not derived from protein breakdown.

The smaller ratios of protein-N to soluble organic-N for the non-chlorophyllous or partly chlorophyllous sections (nos. 1 and 2) than for the chlorophyllous (nos. 3–5) may be attributed to differences in physiological functions. The former composed of meristematic tissues are concerned with growth processes, and the latter, concerned with photosynthesis, require greater amounts of protein for the proteinaceous stroma of the chloroplasts. Another possibility for the lower ratios of protein-N to soluble organic-N in the non-chlorophyllous than chlorophyllous sections may be the greater growth activity of the former which, dependent on cell division, would tend to divide the protein content whereas in the chlorophyllous mature tissues the protein content of the cells would increase by successive depositions.

The association of lower ratio values of protein-N to soluble organic-N with tissues undergoing growth activity is in agreement with the findings of TRAUB (39) and THOMAS (38) who observed that apple twigs, during the plant's most active period of growth, contained higher amounts of amino-N but lower amounts of protein-N and total carbohydrates. Therefore, factors which admittedly affect the velocity of the reaction soluble organic-N $\rightleftharpoons$ protein-N are: (a) stage of plant growth, (b) amounts and kinds of inorganic nitrogen absorbed by roots, (c) conditions affecting carbohydrate synthesis, (d) water and concentrations of essential and accessory nutrient elements, and (e) oxygen tension (37).

Summary

The effects of high *vs.* low nitrogen (140.0 *vs.* 2.8 mg. per liter of nutrient solution) supplied either as NO_3^- or NH_4^+ caused changes in the amounts of the various nitrogenous fractions of the tissues of one-year-old *A. comosus*. The plants of the high-N cultures absorbed approximately five times more nitrogen than of the low-N cultures, although the nitrogen content of the nutrient solutions was fifty times greater for the former than latter cultures.

Total-N and protein-N were from three to four times, and soluble-N was from four to eight times greater in the high-N than low-N cultures. Soluble organic-N was higher in the chlorophyllous than non-chlorophyllous regions of the leaves. Soluble organic-N fractions such as amide, mono-amino and basic were higher in the ammonium than nitrate series. Leaf protein-N was higher in the terminal chlorophyllous than in the basal non-chlorophyllous regions of the leaves.

Protein-N correlated with few exceptions to mono-amino-N and basic-N. The results suggest a continuous formation of amino nitrogen from proteins undergoing normal hydrolysis in the cell or synthesis of proteins by condensation of amino acids in cultures supplied with either adequate or subadequate supplies of inorganic nitrogen. Excessive supplies of inorganic nitrogen either as NH_4^+ or NO_3^- may cause accumulations of soluble organic-N fractions produced by enzymatic synthesis from ammonia with carboxylic acids and similar to those presumably released from protein breakdown.

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THE USE OF 2,3,5-TRIPHENYL-TETRAZOLIUMCHLORIDE AS A MEASURE OF SEED GERMINABILITY[1]

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Seed analysts have long been interested in methods of measuring the germinability of seeds without the necessity of a routine germination test, particularly when dealing with dormant seeds or with seeds requiring a long period for the completion of a test. Even with seeds that can be tested in a week or 10 days it is often desirable to know within a day the general condition of a seed lot. This is especially important in the fall season when it is necessary to know whether or not an early killing frost has caused damage to the seed crop of corn, sorghum, and soybeans.

For dormant seeds, especially the peach, apple, pine, Douglas fir, plum, hawthorn, European mountain ash, witch hazel, and *Rhodotypos kerrioides,* normally requiring a long period of after-ripening, it has been shown by BARTON (1), FLEMION (6, 7, 8, 9) and others that by excising the embryos and placing them on moist filter paper in Petri dishes at room temperature it is possible to determine their vitality within a period of 5 to 10 days. CROCKER and HARRINGTON (2), DAVIS (3), and LEGGATT (13) have published data indicating that the determination of the catalase ratio of dry and germinating seeds may serve as a measure of seed viability. MAR (14) has shown that the amylase activity of soaked oat seed is definitely correlated with germinability. LAKON (10) and others (4, 5, 15) have published data on the use of selenium and tellurium salts in solution as a means of determining the viability of a given seed lot by color reaction of the embryo. LAKON (11, 12) in 1942 compared his results by regular germination test of seeds of oats, barley, wheat, rye, and corn with those obtained by placing the cut seeds in a solution of 2,3-diphenyl-5-methyl-tetrazoliumchloride or 2,3,5-triphenyl-tetrazoliumchloride. The resultant staining of the viable embryos correlated well with his germination results.

Tetrazoliumchloride is colorless but forms carmine red formazans upon reduction. The salt is thus an oxidation-reduction indicator, and the development of the non-diffusible red color in a specific tissue is presumably an indication of the presence of active respiratory processes in which hydrogen radicals are transferred to the tetrazoliumchloride.

In 1945 a supply of 2,3,5-triphenyl-tetrazoliumchloride was obtained by officers of the Chemical Warfare Service in Germany, and Dr. Georg Lakon provided information about the testing program developed by him in which this material was used. Copies of publications, unobtainable in the past,

were received. With the assistance of the junior authors a series of tests was undertaken with seeds of corn (*Zea mays*), barley, (*Hordeum vulgare*), oats (*Avena sativa*), wheat (*Triticum aestivum*), buckwheat, (*Fagopyron esculentum*), cotton (*Gossypium herbaceum*), pea (*Pisum sativum*), rice (*Oryza sativa*), sorgo (*Holcus sorghum*), soybeans (*Soya max*), vetch (*Vicia sativa*), and Bahia grass (*Paspalum notatum*).

Procedure

The use of the tetrazoliumchloride test for corn, barley, oats, rye, and wheat as described by LAKON (11, 12) consisted of soaking seeds to be tested in water for several hours to permit absorption of water and initiation of germination processes. Following the soaking period each seed was bisected longitudinally through the center of the embryo so that each half had a part of the plumule and the radicle. One half of each seed was then placed in a Petri dish and a 1% solution of tetrazoliumchloride poured over the cut seeds until they were completely immersed. The dish was kept for 2 to 10 hours in a dark cabinet at room temperature. At the end of the immersion period each half seed was examined to determine the degree to which the parts of the embryo were stained carmine red. LAKON (11) stated distinct staining of the vital parts of the embryo was evidence of ability to produce a normal seedling. For cereal seeds he concluded that if both the embryo and the scutellum stained carmine red, the seed would be strongly viable. His criterion for seeds of corn (12) was unusual, for he did not consider the primary root in corn absolutely essential to the production of a normal seedling, especially if secondary roots were produced. His view coincides with that of PORTER (16) and also with a report of the Committee on Standardized Tests of the Association of Official Seed Analysts (17). LAKON (12) observed that in the region commonly referred to as mesocotyl, buds were produced on the sides of the region between the plumule and radicle and that in viable seeds these structures stained carmine red, developing into secondary roots. He concluded, therefore, that viable seeds of corn were those which have stained: (1) the entire embryo and scutellum, (2) all parts of the embryo and scutellum including the lateral buds in the mesocotyl region, but not the radicle, or (3) the entire embryo, but not the scutellum; he doubted that all seeds of this group were included among the normal seedlings. In general, it was his belief that a seed possessed stronger vitality if the scutellum stained.

Lakon's procedure was followed with the exception that the concentrations of the solution used were ½%, 1%, and 2% for two kinds of seed, and the length of time for presoaking and immersion in the tetrazolium salt solution was varied for some kinds of seed.

Seed samples used were tested in the Iowa State College Seed Laboratory. In all cases a germination test was made by approved methods to compare with the color-test.

This paper reports the results obtained with the use of tetrazoliumchlo-

ride as a staining solution of the embryos of viable seeds in which germination processes have been initiated.

Results

CORN

For the initial studies with corn a sample with a known germinability of 98% under favorable conditions was selected. Seeds were soaked in water at room temperature from 5:00 P.M. to 8:00 A.M. the following day. Two hundred of the soaked seeds were then cut longitudinally through a median line perpendicular to the embryo side. Care was taken to split the plumule and radicle. One half of each kernel was saved and the entire set of 200 halves covered with a 1% solution of the tetrazoliumchloride. The Petri-dish container was immediately placed in a dark cabinet at room temperature. At the end of 15 minutes a faint pink coloration was noticeable in the embryo region of many seeds. At the end of 2 hours the entire number was examined and 99% showed bright carmine red, not only in the embryo including the plumule and radicle, but also in the scutellar region. The following day 400 more seeds of the same lot were soaked overnight and dipped in boiling water for 5 minutes. Two hundred were then sectioned, covered with the tetrazolium solution, and kept for 8 hours in the dark. No embryos changed color. The remaining 200 seeds were planted in sand, and none germinated. This preliminary test indicated that the tetrazolium solution was capable of differentiating at least between seeds that were nearly 100% germinable and those that were 100% non-viable. Several additional lots of seed corn produced in 1945 were tested with the same solution with results as shown (table I). At the same time a $\frac{1}{2}$% solution was used on one lot as well as a 1% solution. There was no noticeable difference in the results obtained.

TABLE I

COMPARATIVE DETERMINATION OF SEED CORN VIABILITY BY APPROVED METHODS OF GERMINATION AND BY STAINING REACTION WITH 1% SOLUTION OF TETRAZOLIUMCHLORIDE

SAMPLE NO.	STAINING		VIABILITY IN SAND	
	NO. OF SEEDS	COLORATION	NO. OF SEEDS	GERMINATION
		%		%
5	200	90.0	200	90.0
22	200	90.0	200	89.5
23	200	86.5	200	90.5
39	200	22.5	200	23.5
40	200	9.0	200	7.0
1036	400	75.0	400	70.0
1072	400	86.2	400	82.0
1127	400	99.0	400	98.0
1169	400	85.0	400	81.0
1223	400	99.2	400	99.0
1228	400	97.5	400	90.0
1253	400	75.8	400	69.0
1298	400	90.8	400	90.0

SMALL GRAINS, SORGHUM, BUCKWHEAT, AND LEGUMES

Samples of barley, oats, wheat, sorgo, buckwheat, popcorn, peas, soybeans, and vetch were tested with tetrazolium solution in much the same manner as described for corn. The soaking period, both in water and in the solution, was varied. The seeds of small grain, sorgo, popcorn, and buckwheat were cut medianly through the long axis of the embryo. The seeds of pea, soybean, and vetch were cut between the cotyledons, bisecting the radicle longitudinally. The data (fig. 1) show that for 3 lots of barley, 2 lots of oats, wheat, buckwheat, soybean, and popcorn there was a close relationship between the percentage of stained embryos and of normal sprouts produced in the ordinary laboratory germination test in sand (table II).

The tests with sorgo indicated that the color reaction might not be entirely reliable for the seeds of this plant; however, more tests are necessary before proof can be established. Sorgo seed would probably respond in a manner similar to that of corn and cereals. A careful study of the degree of coloration of the embryo, as well as time of soaking in the solution, may result in a refinement of the method.

Seeds of legumes require different treatment than those of grasses. For example, a long period of soaking in the chloride solution results in a coating of reddish stain over the entire flat surface of each cotyledon, and the stain must be rubbed off before the radicle can be examined. Furthermore, there is no indication as to the condition of the epicotyl in peas and vetch seed or of the plumule in soybean seed, primarily because it is difficult to split the plumule or epicotyl longitudinally in the sectioning process. To determine by this method which bean seeds would produce "baldhead" seedlings is practically impossible. The primary root emerges early in the germination of legume seeds and before the epicotyl or plumule shows much growth. This condition may prevent any practical use of the staining method as a means of determining the germinability of such seeds. Further investigations are needed before conclusions can be drawn.

FIG. 1. Longitudinal sections of seeds showing area (heavy black or shaded portions) that stained carmine red when soaked in a $\frac{1}{2}\%$ solution of 2,3,5-triphenyl-tetrazoliumchloride: a, procambial strand; b, coleoptile; c, adventitious root; d, primary root; e, plumule. Figure 1, 20 ×; figure 6, 12 ×; others, 6 ×.

1, 2, 4, 5, 6. Sections of Bahia grass, barley, oat, rice, and wheat seeds. Only those which showed the entire embryo and scutellum with carmine red stain were considered viable.

3. Corn kernel—seeds which stained carmine red in the scutellum and plumule regions down to and including the lateral root buds were considered viable. The stain was usually present throughout the scutellum and embryo.

7. Pea cotyledon—a section cut between the cotyledons and through the radicle. Those with a carmine red radicle were considered viable although no evaluation of the condition of the epicotyl could be made.

8. Soybean cotyledon—a section cut between the cotyledons and through the radicle. Those with a carmine red radicle were considered viable, although no evaluation of the condition of the plumule could be made.
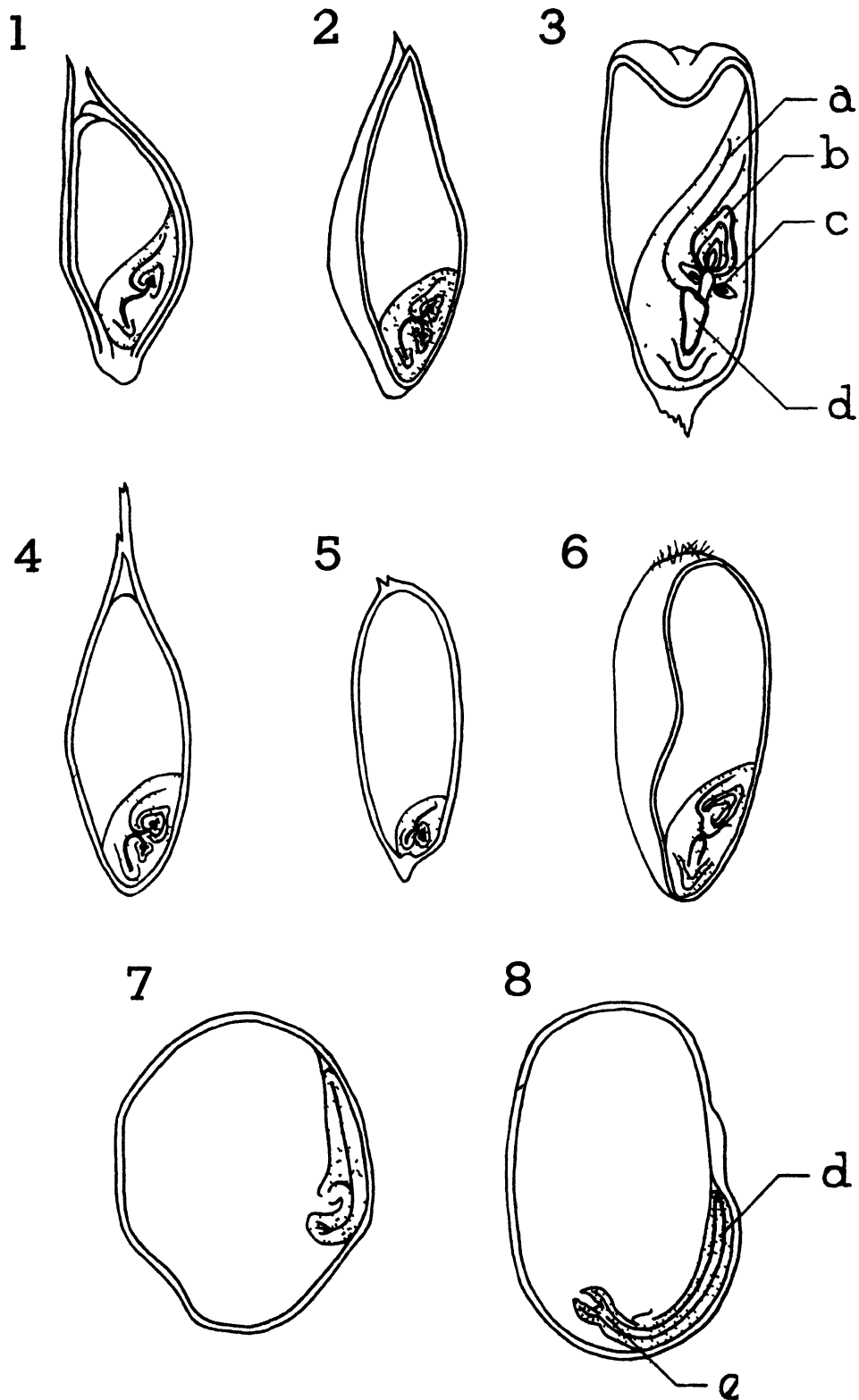
BAHIA GRASS

Determination of viability in seeds of Bahia grass is one of the most difficult problems in seed technology. Recommended procedures are to dehull the fresh fruit seed, to scrape lightly with a scalpel, and moisten with 0.1% potassium nitrate solution. These procedures are not entirely satisfactory as dehulling is a very tedious process and nitrate solution usually results in an abundance of fungous growth around each seed, making evaluation of sprouting very difficult.

TABLE II

COMPARATIVE DETERMINATION OF SEED VIABILITY BY LABORATORY GERMINATION AND BY STAINING REACTION WITH 1% SOLUTION OF TETRAZOLIUMCHLORIDE

SAMPLE		STAINING TEST				LABORATORY GERMINATION	
		NO. SEEDS	COLORED	STAINING TIME		NO. OF SEEDS	NORMAL SPROUTS
			%	hrs.	min.		%
Barley	1A*	200	90.0	5		200	93.0
,,	1A†	200	63.0	5		200	80.5
,,	27C*	200	75.0	5		200	74.0
,,	27C†	200	61.0	5		200	60.5
Buckwheat	1	200	98.5	4		200	95.0
Oats	36	179	83.2	2		200	94.5
,,	38	200	75.0	2		200	89.5
,,	Marion	200	94.0	1		400	98.0
,,	Tama	200	47.0	1		400	50.0
Peas	55101	194	90.2		20	200	81.5
,,	55105	192	91.6		20	200	78.5
,,	55106	197	90.3		20	200	85.0
Popcorn	43610	200	32.0	2		200	27.5
Wheat	61	200	84.0	5		200	85.0
,,	120	190	73.6	5		200	71.5
Soybean	1	200	100.0		30	200	96.0
Sorgo	9466	200	93.5	2		200	76.0 (80)‡
,,	9467	200	75.5	2		200	49.0 (68)‡
,,	49545	200	69.5	2		200	45.0 (51)‡
Vetch	1	200	58.0		15	200	75.5

* New crop.
† Old seed.
‡ Seed treated with Arasan.

Two types of tests using tetrazoliumchloride solution were undertaken. The first consisted of soaking the seeds from a pure seed fraction in water at 20° to 35° C. for 1 to 7 days. At the end of each day 3 lots of 50 seeds each were removed, sectioned, and soaked in $\frac{1}{2}$%, 1%, and 2% solutions of tetrazoliumchloride. The results showed that a 2% solution was too strong, that there was little difference between the results obtained with a $\frac{1}{2}$% or a 1% solution, and that after 3 days of soaking the percentage of colored embryos declined. The declining of the colored embryos suggests that seeds of Bahia grass, if soaked several days, begin to lose vitality. Such a response is not unexpected, because many seeds will not germinate in water.

The seeds absorb water and germination is initiated, but, if kept too long, decomposition occurs.

The second type of test with Bahia grass consisted of soaking the seeds 16 hours in distilled water at 20° C. The seeds were then placed in a Petri dish on moist filter paper and kept in a germinator with an alternating temperature of 20° C. for 16 hours and 35° C. for 8 hours. Immediately after the initial period of soaking and at subsequent intervals of 24 hours, 50 seeds were removed, sectioned, and placed in a 1% solution of tetra-zoliumchloride. At the end of 8 and 24 hours the sections were examined and classified as light-stained, dark-stained, viable, and non-viable. Only those with the entire embryo and scutellum stained light or dark were considered viable (table III).

TABLE III

RESULTS OF SOAKING 50 CUT SEED SECTIONS OF BAHIA GRASS IN 1% SOLUTION OF TETRA-ZOLIUMCHLORIDE FOR 8 AND 24 HOURS*

NO. OF DAYS AFTER SOAKING	NO. OF DARK-STAINED SEEDS	NO. OF LIGHT-STAINED SEEDS	VIABLE	NON-VIABLE	TIME IN SOLUTION
			%	%	hrs.
0	2	48	100	0	8
	35	15	100	0	24
1	8	42	96	4	8
	37	13	96	4	24
2	29	20	96	4	8
	44	5	94	6	24
3	35	15	100	0	8
	47	3	98	2	24
4	22	24	88	12	8
	42	3	90	10	24
5	25	25	100	0	8
	40	10	100	0	24
6	31	19	96	4	8
	38	9	94	6	24

* Seeds were soaked 16 hrs. in water at 20° C., kept at temperatures alternating between 20° and 35° C. for period of 1 to 6 days, and sectioned.

In all cases the percentage of dark-stained embryos increased markedly as the period of immersion was increased from 8 to 24 hours. Since the embryos not deeply stained were colored throughout the scutellar region as well as in the embryo proper, they were considered viable. Among the lots kept in the germinator for 1 to 6 days and soaked 24 hours in the chloride solution, the differences in viability, on the basis of 50 seeds, were not significant. On the other hand, the large percentage of dark-stained seeds retained in the germinator for 2 to 4 days after soaking, indicated that such a procedure may be preferable. The viability by this method of this particular lot of Bahia grass seed appeared to be between 90% and 100%.

A germination test of this lot was made as follows: 100 seeds were dehulled, soaked 5 minutes in a 25% solution of chlorox, placed on moist filter papers in a Petri dish, and kept in a germinator with alternating tempera-

tures of 15° C. for 16 hours and 30° C. for 8 hours. At the end of 9 days all the seeds had produced strong, normal sprouts. Another sample of 200 seeds, treated in the same way except that the temperature alternated from 20° to 35° C., gave 95% germination in 5 days. The results indicated that the viability of this lot of seed was nearly 100% which agrees with the index of viability as measured by the tetrazoliumchloride method. They also suggested that alternating temperatures of either 15° to 30° C. or 20° to 35° C. were favorable for the germination of Bahia grass seed.

RICE SEED

Two hundred seeds from each of four varieties of rice were soaked in water for 16 hours at room temperature. Each seed was sectioned longitudinally through the embryo, and one half of each placed in a 1% solution of tetrazoliumchloride for 4 hours. At the end of the soaking period the

TABLE IV

COMPARATIVE DETERMINATION OF THE VIABILITY OF RICE SEED BY STAINING WITH 1% SOLUTION OF TETRAZOLIUMCHLORIDE AND LABORATORY GERMINATION BASED ON 200 SEEDS TESTED

VARIETY	VIABILITY BY STAINING	LABORATORY GERMINATION		
		BETWEEN BLOTTERS	BETWEEN BLOTTERS 20°–30° C.	SAND
	%	%	%	%
Nira	66.0	65.5	55.0	70.0
Fortuna	87.0	84.0	77.5	81.0
Prelude	84.5	75.5	72.0	71.5
Zenith	94.5	90.0	78.0	86.5

sections were examined under a binocular microscope and all those with the embryo and scutellum stained light or dark red were classed as viable. Three lots of 200 seeds each from the 4 varieties were prepared and tested for germination by three methods: (1) between blotters, (2) presoaked 16 hours and planted between blotters, and (3) presoaked 16 hours and planted in sand. All tests for germination were made at 20° C. for 16 hours and 30° C. for 8 hours each day for 10 days (table IV). With the exception of the Prelude variety soaking before planting resulted in a higher percentage of germination, and germination in blotters or sand of presoaked seed agreed with the embryo color reaction.

COTTON SEED

A few attempts were made to apply the tetrazolium color test to cotton seed. One difficulty was that some seeds after soaking overnight were hard and could not be sectioned. Such seeds presumably would not have absorbed water, and germination processes could not have been initiated. Many seeds that did absorb water when sectioned and soaked in a 1%

tetrazoliumchloride solution stained readily in the embryo region; but the seed coats separated easily from the embryos and cotyledons, and the latter further separated from each other.   An evaluation of the color test, therefore, was difficult to make.

Discussion and summary

Determination of living embryos of seeds by means of the color indicator 2,3,5-triphenyl-tetrazoliumchloride as recommended by LAKON (12) was investigated, using seeds of several members of the grass family, three leguminous species, buckwheat, and cotton.   A comparison of the percentage of stained embryos in a given lot with the percentage of normal sprouts obtained in a standard germination test showed close agreement in 13 samples of corn, two samples each of wheat and oats, three samples each of barley and rice, and one sample each of buckwheat, popcorn, soybean, peas, and Bahia grass.   The comparisons for vetch, sorgo, two samples of oats and peas, and one sample of barley were not in close agreement, yet the differences in results shown by the two methods were not great.

With the exception of corn the criterion used for classifying grass seeds as viable was the almost complete staining of the embryo proper and scutellum.   Absence of color in the radicle below the mesocotyl region or in the lower half of the scutellum, however, was not considered sufficient grounds for classification of a corn seed as non-viable.

In seeds of pea, soybean, and vetch it is impossible to section through the epicotyl or plumule region regularly.   The only cut part, therefore, with which the solution can readily make contact, is the radicle.   Development of a radicle in leguminous seeds without consideration of the plumule or epicotyl is insufficient evidence for classification as a normal seedling. For that reason the method investigated may not be applicable to seeds producing baldheads when they germinate.

The method appears to have some merit for seeds of Bahia grass which are difficult to germinate.   It is recommended that the hulls be removed before placing the seeds to germinate.   This is a tedious and time-consuming process.   By the staining method it was possible to soak the seeds 16 hours, place them on a moist substratum at 20° to 35° C. for 3 or 4 days, cut them into sections, and place in the tetrazoliumchloride solution for 24 hours.   More samples must be tested by this method before definite conclusions can be stated.   When the hulling method is used it is desirable to soak the seeds in a 25% solution of chlorox to prevent the growth of surface molds.

A standard method of germination may not be the one that measures the true germinability of a particular kind of seed.   For example, in the tests with four varieties of rice the standard method failed to measure the maximum germinability of the seed of three varieties.   When the seeds were soaked and planted in sand or blotters, the percentage germination compared closely with the percentage of stained embryos that were soaked in the tetrazoliumchloride solution.

As pointed out in the introductory statement, tetrazoliumchloride is colorless but forms carmine red formazans upon reduction. The salt is an oxidation-reduction indicator, and the development of the non-diffusible red color in a specific tissue is presumably an indication of the presence of active respiratory processes in which hydrogen radicals are transferred to the tetrazoliumchloride. If further research should establish this hypothesis, the process should prove to be of general value in physiological experiments as well as an index of germinability of seeds. Because the measurements of "germinability" are indirect, however, and dependent upon all of the factors affecting the oxidation-reduction potential of the cells, careful standardization of procedure will probably be necessary.

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CHLOROPHYLL AND PROTEIN INTERRELATIONSHIPS IN *ANANAS COMOSUS* (L.) MERR.[1]

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(WITH FOUR FIGURES)

Studies on a quantitative separation and analysis of chloroplastic matter by different investigators have revealed that chlorophyll is held by chemical forces on proteinaceous matter. This matter, named phyllochlorin by MESTRE (17), chloroplastin by STOLL (37), photosynthin by FRENCH (8), and chloroglobin by RABINOWITCH (21), is presumably identical with the substance composing the stroma of chloroplasts.

FREY-WYSSLING (9), discussing the composition of chloroplasts, stated that, according to GUILLERMOND, MANGENOT, and PLANTEFOL (13) and SHARP (24), the structure of the chloroplasts of higher plants is homogeneous and not granular with small green particles imbedded in a colorless stroma, as postulated by MEYER (18) and SCHIMPER (22), and that the granulations are artifacts. According to HEITZ (15), the grana are not spheres but disks oriented parallel to the surface of the disk-shaped particles which alone contain the chlorophyll. NOACK (20) observed that colloidal chlorophyll does not show fluorescence except when adsorbed in monomolecular layers. Also, EULER *et al.* (7) have reached a similar conclusion from determinations of the quantity of chlorophyll in one single plastid.

Methods

The isolation of chloroplasts or of chloroplastic matter has been effected in various ways by different investigators (3, 5, 8, 11, 12, 14, 17, 19) and with somewhat similar results.

ISOLATION OF CHLOLOPLASTIC MATTER

In former studies (25) the leaves of *A. comosus* were segregated into groups represented by old (B), mature (C), active (D), and young (E) leaves. In *A. comosus,* as in all monocotyledonous plants, the old (B) leaves occupy positions at the basal end of the stem, the young (E) at the apical end, and the mature (C) and active (D) at the medial section. Such leaves are cut for many studies into four or five cross sections in order to segregate tissues of different stages of development and physiological function. The basal sections (no. 1) of such leaves are lacking in chlorophyll, and in leaves which have not completed growth they are composed mostly of meristematic tissues. The chlorophyllous region of the blade is divided into terminal (no. 5), medial (no. 4) and basal (no. 3) sections and the neck

or region between the non-chlorophyllous (no. 1) and chlorophyllous blade (nos. 3, 4, 5) is known as the transitional section (no. 2).

In this study the isolation of chloroplastic matter was made as follows: One hundred grams of tissues from the chlorophyllous section (no. 4) of freshly collected active (D) leaves of *A. comosus* var. smooth Cayenne (25) cut approximately 2 mm. wide were placed with 250 ml. of 0.06 N NaOH in a Waring mixer and macerated at high speed for ten minutes. The mixture was squeezed by hand pressure through 200-mesh silk cloth or Canton flannel. The residue in the cloth was wetted with 25 ml. of 0.06 N NaOH and squeezed at successive intervals until all green color was removed from the residue.

The residue was discarded and the extract placed in 100 ml. "oil" centrifuge tubes and centrifuged for the first 5 minutes at about 750 r.p.m., the next 2 minutes at about 1100 r.p.m., and the last 3 minutes at about 1500 r.p.m. The gradual increases of centrifugal speed caused starch to settle at the bottom of the container with the least admixture of chloroplastic matter, while the latter remained in the supernatant liquid.

The supernatant liquid, containing chloroplastic matter, was transferred to clean centrifuge tubes without disturbing the precipitated starch, treated with acetic acid to pH 4.0 (1 ml. or more of 6 N glacial acetic acid), agitated gently to effect mixing, and then centrifuged at different speeds as mentioned. The supernatant liquid was decanted and the precipitate examined carefully for starch granules in the bottom layer of the container. When starch was detected, the precipitate was treated with 75 ml. 0.06 N NaOH, agitated to effect uniform suspension of the precipitate, and centrifuged as described. The supernatant liquid containing chloroplastic matter was removed and the precipitated starch discarded. The chloroplastic matter was flocculated with acetic acid as before and centrifuged.

To facilitate transferring the chloroplastic matter from the tubes to volumetric flasks, 1 ml. of 0.06 N NaOH was added, the contents agitated and poured into the flask. The alkaline suspension was acidified with acetic acid to pH 4.5, the flask filled to the mark with H_2O and placed in the refrigerator. The concentration of the contaminant sodium acetate could be reduced by removal of the clear supernatant liquid at daily intervals and replacement with H_2O. The mixture was agitated before withdrawing samples for analysis.

Chlorophyll was extracted from the chloroplastic matter with acetone and determined in a Klett-Summerson photoelectric colorimeter using a light filter no. 60. The proteinaceous residue was analysed for total nitrogen.

Results

ANALYSES OF CHLOROPLASTIC MATTER

Analyses of the chloroplastic matter from the leaves of *A. comosus* yielded 6.5 mg. of chlorophyll and 22.3 mg. of nitrogen per 20 grams of fresh tissue, the latter being equivalent to 140.0 mg. of protein (22.3×6.25).

HANSON (14) claimed that one tetrad of chlorophyll molecules (M.W. $900 \times 4 = 3600$) is associated in the cell with one molecule of chloroplastic globulin (M.W. 68,000). Thus Hanson's estimated ratio of chlorophyll to protein is equal to 0.0530, *i.e.*, $3600 \div 68,000$, whereas that obtainable in this study was 0.0465, *i.e.*, $6.5 \div 140.0$, the latter being 14.0% lower than the theoretical. The difference between Hanson's theoretical ratio and that obtained in our study may be due in part to incomplete extraction of chlorophyll—the extract was estimated to contain approximately 95% of the total chlorophyll in the chloroplastic matter because the latter was faint greenish yellow after repeated extractions.

ANALYSES OF TISSUES WITHOUT EXTRACTION OF PROTOPLASMIC MATTER

Chlorophyll/protein ratios from other studies (26, 27) show gradients of ascending values from the basal to the terminal sections of leaves (table I). It will be noted that the ratios of chlorophyll to protein for section no. 4 of the active (D) leaves (25) were greater in the studies referred to above, where no separation of the chloroplastic matter from the tissues was effected but protein-N was estimated as the difference between total-N and soluble-N, than the value 0.0465 in the present study or the theoretical value 0.053. The results in table I indicate that the amounts of protein in relation to chlorophyll were about one-half as great as in the isolated chloroplastic matter and suggest a low rate of protein synthesis, a high rate of protein breakdown, or a low rate of chlorophyll reduction.

In sections no. 2 and no. 3, which are chronologically and physiologically less advanced than no. 4 and no. 5, the protein content of the tissues in relation to chlorophyll was less, either because of shorter periods of deposition and differences in the physiological functions of the tissues or of greater amounts of extracted protein, due to high pH values in sections no. 2 and no. 3 than in no. 4 and no. 5, which reduced the degree of association of protein with chlorophyll (23).

In general protein correlated with the chlorophyll content of tissues (figs. 1, 2, 3, and 4). The coefficient of correlation, calculated according to SNEDECOR (34) from $r = Sxy/\sqrt{(Sx^2)\ (Sy^2)}$ was statistically significant under experimental conditions which were favorable to plant growth. Chlorophyll/protein ratios in many chlorophyllous sections were in agreement with the theoretical ratio but in others, mostly partly chlorophyllous, they were either higher or lower, presumably because of the methodological shortcomings stated above.

From previous publications (27, 28), depicting relationships between chlorophyll and protein in cultures supplied with equal amounts of nitrogen as NO_3 or NH_4 but with different amounts of iron, correlation between chlorophyll and protein in all cultures is shown (fig. 1). The coefficient of correlation for the minus-iron cultures in the ammonium series was better than for the minus-iron cultures in the nitrate series, because the intake of iron, which occurred as impurity in the C.P. salts of the nutrient solu-

TABLE I

PROTEIN-N, CALCULATED PROTEIN (PROTEIN-N × 6.25) AND CHLOROPHYLL OF FRESH TISSUE; ALSO, CHLOROPHYLL/PROTEIN RATIOS IN DIFFERENT LEAF SECTIONS OF ONE-YEAR-OLD *Ananas comosus*

LEAF SECTIONS		NITRATE SERIES*				AMMONIUM SERIES†			
		PROT.-N	PROTEIN	CHLOROPHYLL	$\frac{CHLOR.}{PROT}$	PROT.-N	PROTEIN	CHLOROPHYLL	$\frac{CHLOR.}{PROT.}$
		mg./gm.	*mg./gm.*	*mg./gm.*		*mg./gm.*	*mg./gm.*	*mg./gm.*	
Mature (C)	1	0.12	0.75			0.38	2.38		
	2	0.37	2.31	0.35	0.151	0.86	5.37	0.50	0.093
	3	0.80	5.00	0.59	0.118	1.66	10.38	0.83	0.080
	4	1.00	6.25	0.69	0.110	2.14	13.38	1.05	0.078
	5	1.20	7.50	0.86	0.114	2.50	15.63	1.22	0.078
Active (D)	1	0.19	1.19			0.34	2.12		
	2	0.31	1.94	0.28	0.144	0.61	3.81	0.30	0.079
	3	0.62	3.88	0.46	0.119	1.45	9.05	0.56	0.062
	4	1.22	7.62	0.60	0.079	2.10	13.13	0.78	0.059
	5	1.58	9.87	0.74	0.075	2.48	15.50	1.02	0.066
Young (E)	1	0.39	2.44			0.33	2.06		
	2	0.39	2.44	0.12	0.049	0.64	4.00	0.20	0.050
	3	0.66	4.12	0.33	0.080	1.19	7.44	0.40	0.054
	4	1.25	7.82	0.50	0.064	1.92	12.00	0.78	0.065

* Grown in solution cultures supplied with nitrate.

† Grown in solution cultures supplied with ammonium salts.

tions, was greater from the ammonium-N series with ultimate lower pH values than from the nitrate-N series with higher ultimate pH values than at the beginning of the absorption period.
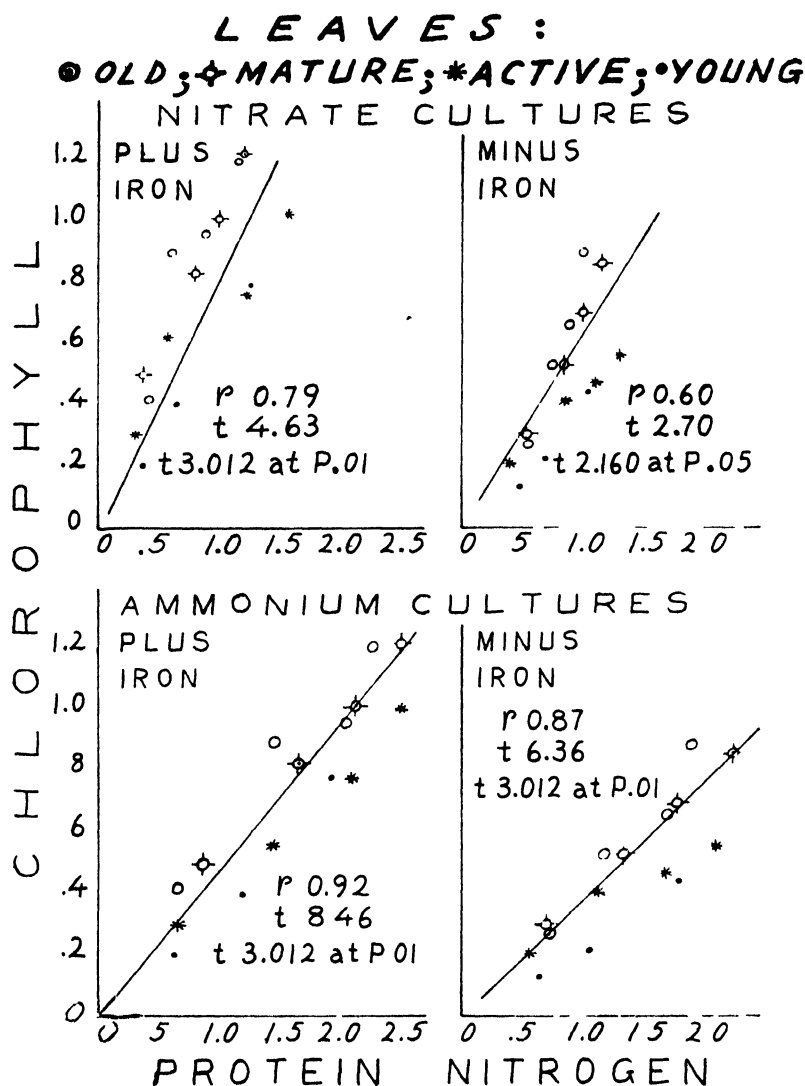

FIG. 1. Correlation coefficient, *r*, and its statistical significance for chlorophyll and protein in different sections of the leaves of different ages of *A. comosus* grown in solution cultures supplied with or without iron and with equal amounts of nitrogen either as NO_3^- or NH_4^+.

From previous publications (**31, 32**), pertaining to the effects of 140.0 *vs.* 2.8 mg. of nitrate or ammonium nitrogen in nutrient solutions on the chlorophyll and protein content of different leaf sections, highly significant correlation coefficient values are shown (fig. 2). In this figure as in figures 1, 3, and 4, each point represents the mean value of similar sections in the different leaf groups instead of individual sections. It will be noted that protein synthesis for the low-N cultures was greatly inhibited by nitrogen

deficiency, which also caused a parallel reduction in the chlorophyll content of the tissues (fig. 2).

From previous publications (**28, 29**), depicting the effects of 205 *vs.* 4 mg. of potassium per liter of nutrient solution on the chlorophyll and protein content of different leaf sections, it is shown that correlation coefficient values were highly significant for all cultures and may suggest that potassium played no direct role in protein or chlorophyll synthesis (fig. 3).
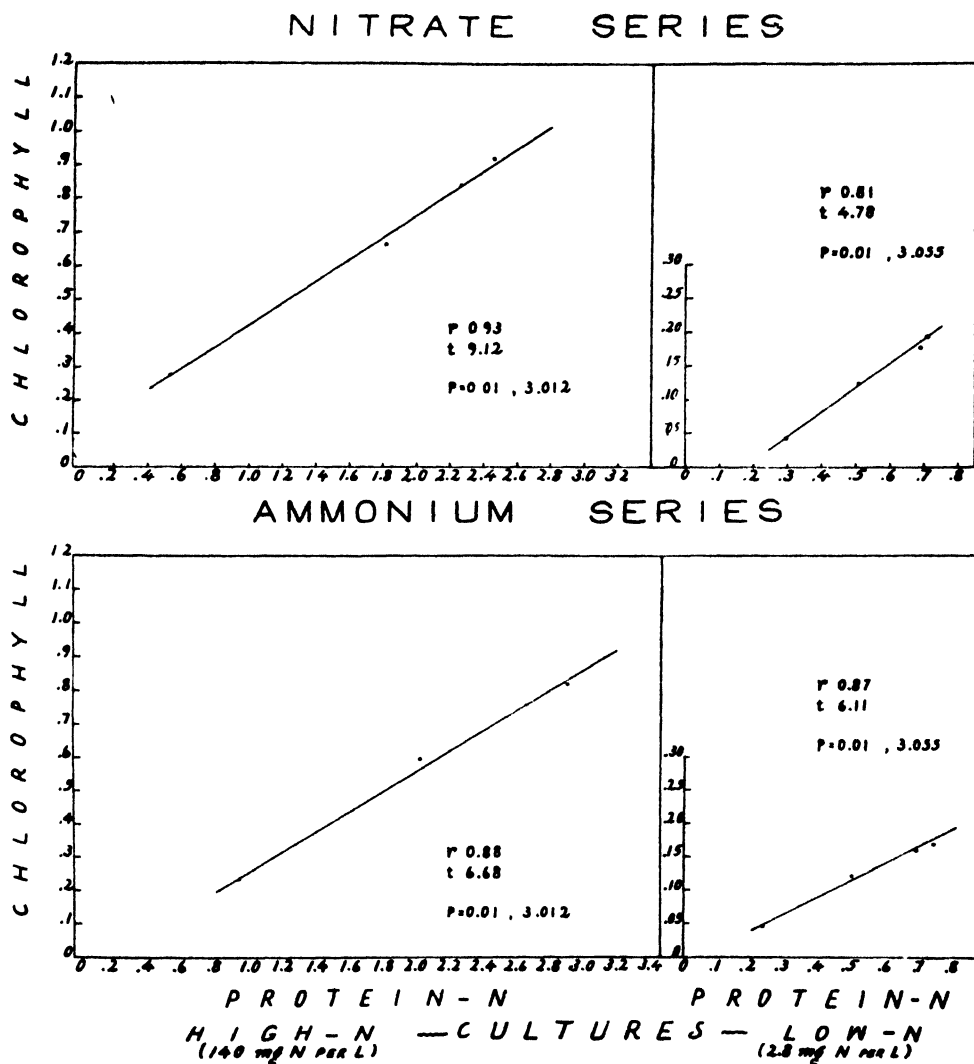

FIG. 2. Correlation coefficient, *r*, and its statistical significance for chlorophyll and protein in different sections of the leaves of different ages (the values are means for similar sections of leaves of different ages) of *A. comosus* grown in solution cultures with 140.0 or 2.8 mg. of nitrogen per liter, supplied either as NO_3^- or NH_4^+.

From a previous publication (**25**), depicting chlorophyll and protein relationships in two shoots from the same plant, of which one was exposed to seasonal light and the other was covered by a bag of black cloth for 56 days, statistically significant correlation coefficient values for the exposed

plant at $P = 0.05$ level but not at $P = 0.01$ level are indicated (fig. 4). Correlation between chlorophyll and protein was lacking for the covered plant, presumably because darkness inhibited chlorophyll formation. It may be noted, however, that some sort of relationship existed between chlorophyll and protein in the old (B) and in the mature (C) leaves which at the time
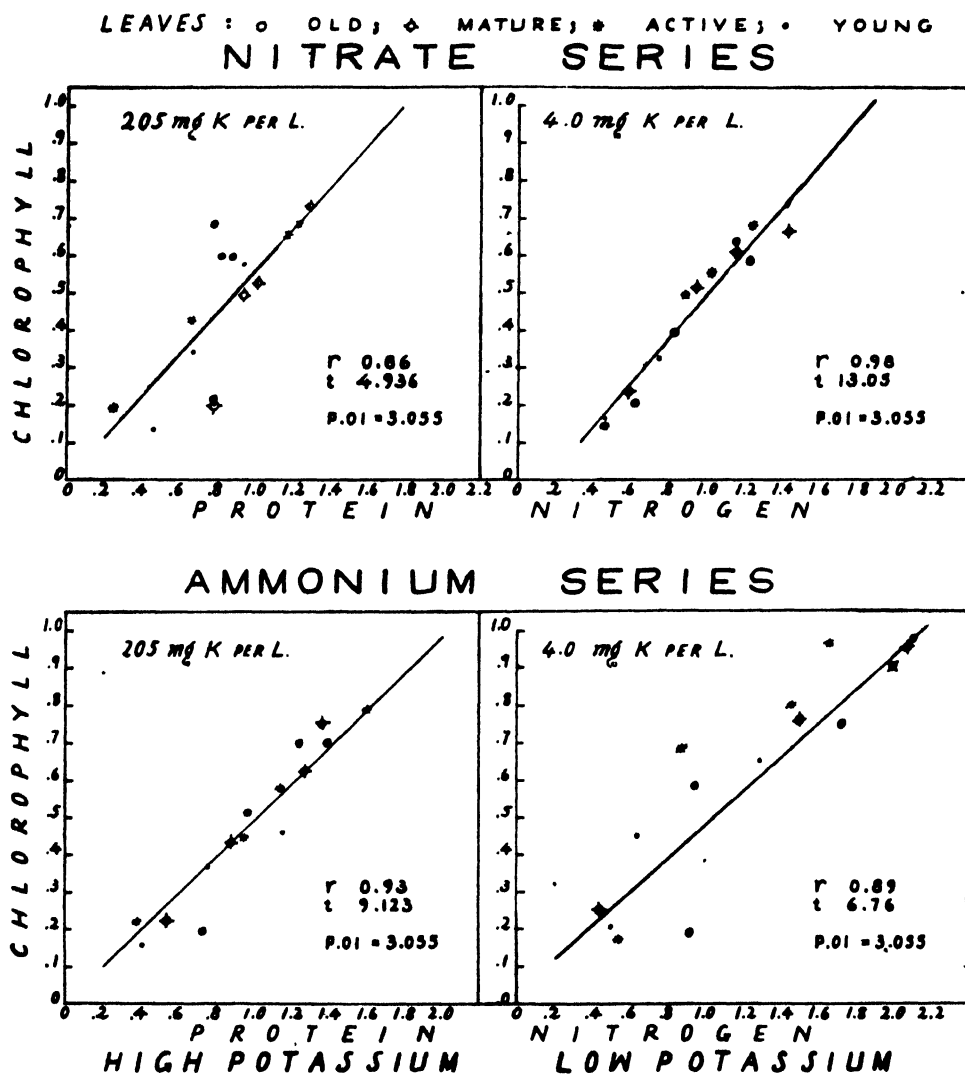

FIG. 3. Correlation coefficient, r, and its statistical significance for chlorophyll and protein in different sections of the leaves of different ages of *A. comosus* grown in solution cultures with 200 or 4 mg. of K per liter and supplied with equal amounts of nitrogen as NO_3^- or NH_4^+.

of covering had approximately completed growth, but not in the active (D) and young (E) leaves which developed in darkness, lacked chlorophyll, and contained relatively little protein-N.

The findings in table II suggest that in cultures of *A. comosus* supplied with nitrate as the source of inorganic nitrogen, protein deposition in the

TABLE II

PROTEIN-N, CALCULATED PROTEIN (PROTEIN-N × 6.25) AND CHLOROPHYLL OF FRESH TISSUE; ALSO, CHLOROPHYLL/PROTEIN RATIOS IN DIFFERENT LEAF SECTIONS OF ONE-YEAR-OLD *Ananas comosus* SISTER SHOOTS

LEAF SECTIONS		EXPOSED*				COVERED†			
		PROT.-N	PROTEIN	CHLOROPHYLL	CHLOR./PROT.	PROT.-N	PROTEIN	CHLOROPHYLL	CHLOR./PROT.
		mg./gm.	*mg./gm.*	*mg./gm.*		*mg./gm.*	*mg./gm.*	*mg./gm.*	
Mature (C)	1	0.368	2.30	.....	.....	0.245	1.53	.....	.....
	2	0.502	3.14	0.071	0.0226	0.350	2.19	0.017	0.0078
	3	0.646	4.03	0.220	0.0546	0.394	2.46	0.078	0.0318
	4	0.870	5.44	0.320	0.0589	0.516	3.23	0.075	0.0232
	5	0.870	5.44	0.272	0.0500	0.525	3.28	0.102	0.0310
Active (D)	1	0.313	1.95	0.000	.....	0.327	2.04	0.000	.....
	2	0.340	2.12	0.035	0.0165	0.310	1.94	0.000	.....
	3	0.438	2.74	0.126	0.0460	0.348	2.17	0.000	.....
	4	0.682	4.26	0.128	0.0300	0.464	2.90	0.007	0.0024
	5	0.885	5.54	0.294	0.0530	0.464	2.90	0.082	0.0280
Young (E)	1	0.400	2.50	0.000	.....	.....	.....	0.000	.....
	2	0.317	1.98	0.038	0.0192	.....	.....	0.000	.....
	3	0.514	3.22	0.048	0.0149	0.347	2.16	0.000	.....
	4	.....	.....	.....	.....	0.206	1.29	0.020	0.0155

* Exposed to seasonal sunlight.
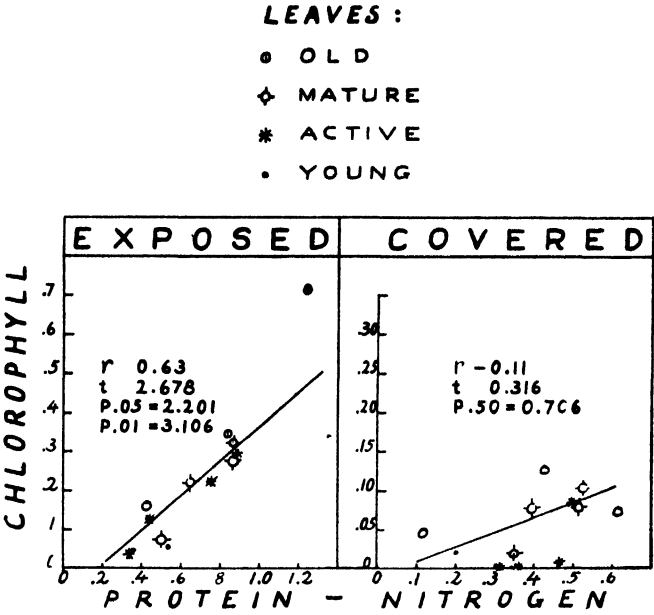† Covered with a black cloth for 56 days.

FIG. 4. Correlation coefficient, r, and its statistical significance for chlorophyll and protein in different sections of the leaves of different ages of *A. comosus* sister shoots grown on the mother plant, of which one was kept in darkness for 56 days by covering with a black cloth; and the other was exposed to seasonal light.

chlorophyllous sections (nos. 3, 4, and 5) was in much greater amounts than in the basal non-chlorophyllous sections (no. 1). This may indicate that protein deposition was simultanteous with chlorophyll production, presumably because of nitrate reduction and assimilation and carbohydrate synthesis in these sections.

In variegated leaves of *Ananas bracteatus* less protein-N was present in the non-chlorophyllous than chlorophyllous areas and the ratios of chlorophyll to protein were generally lower in the former than latter areas (table III). Chlorophyll to protein ratios in the green areas of leaves no. 2 and

TABLE III

CHLOROPHYLL AND PROTEIN-N OF FRESH TISSUE AND CHLOROPHYLL TO PROTEIN (PROTEIN-N × 6.25) RATIOS IN THE NON-CHLOROPHYLLOUS OR CHLOROPHYLLOUS AREAS OF VARIEGATED LEAVES OF *Ananas bracteatus* GROWN UNDER FIELD CONDITIONS

LEAVES		CHLOROPHYLL	PROTEIN-N	PROTEIN	CHLOR.
No.	COLOR				PROT.
		mg./gm.	*mg./gm.*	*mg./gm.*	
1	White	0.068	0.28	1.75	0.039
1	Green	0.301	1.11	6.93	0.043
2	White	0.086	1.34	9.37	0.010
2	Green	0.729	2.13	13.30	0.055
3	White	0.083	0.81	5.06	0.016
3	White and green	0.231	1.07	6.67	0.035
3	Green	0.351	1.03	6.43	0.055

no. 3 had attained the theoretical value of 0.055, but in leaf no. 1 the observed ratio was about 22% lower than the theoretical value.

Discussion

STILES (36), SPOEHR (35), RABINOWITCH (21), WEIER (38), BURK-HOLDER (4), GAFFRON (10), and FREY-WYSSLING (9) have presented the various aspects of photosynthesis insofar as it concerns chlorophyll and chloroplastic proteins either as a coordinating system or as independent systems.

Sugar synthesis in green plants by photosynthetic activity is effected by reduction of carbon dioxide with evolution of oxygen. Carbon dioxide reduction takes place in other organisms lacking chlorophyll which may or may not be sensitive to light, according to GAFFRON (10) and WERKMAN and WOOD (39), suggesting that the proteinaceous matter in the cell (which may function similarly to the proteinaceous stroma in the chloroplasts), and not the chlorophyll is the locus for carbon dioxide reduction. Experimental evidence (6) on the time required for the photosynthetic mechanism corroborates the view that carbon dioxide reduction may be effected in the absence of chlorophyll proper in some other adjacent region of the system and possibly in the chloroplastic stroma. HÖBER (16), in an attempt to explain certain observations on studies in photosynthesis, states his point of view as follows: "The time element, possibly associated with long range forces, may be necessary to explain the observed fractional quantum efficiency for a large assembly of chlorophyll molecules in the interior of the chloroplast to absorb four light quanta simultaneously and then to transmit the electronic excitation energy from one molecule to the next by some sort of resonance effect, until it reaches a point on the surface occupied by an adsorbed CO_2 molecule."

The data on protein-N and chlorophyll content in various leaf sections of *A. comosus*, in previous publications (27, 28, 29, 30, 31, 32), indicate in general gradients which increased from the basal chlorophyll-free semi-meristematic no. 1 section to the terminal more or less senescent no. 5 sections. In plants supplied with insufficient amounts of iron, nitrogen, or light the chlorophyllous sections of the leaves contained relatively equal or slightly greater amounts of protein-N than the non-chlorophyllous basal no. 1 sections. Inhibition of chlorophyll synthesis by deficiencies in iron, light, or nitrogen caused a parallel reduction of protein depositions in the chlorophyllous tissues.

In plants adequately supplied with iron, light, or nitrogen, chlorophyll to protein ratios were approaching the theoretical value, although they fluctuated considerably for different sections of the leaves. Lowest ratios and ratios nearer the theoretical value were obtained mostly in the distal more mature regions of the leaves, *e.g.*, sections 4 and 5, but in the proximal sections 2 or 3 which are less mature, the ratios were ordinarily higher. It is probable, therefore, that protein depositions in the latter were either,

limited by unknown physiological causes or that some protein **might have** been incorporated in the soluble organic-N fraction and the reported protein values were not sufficiently high to yield the theoretical ratio, 0.055. It was observed in subsequent studies, which will be reported elsewhere, that from 12.0% to 28.0% of the total water-extractable nitrogen is protein-aceous and may be precipitated by acetic acid, while from 1.0% to 12.0% is proteose-N which is precipitable by trichloroacetic acid. In many analyses reported above, all proteose-N was incorporated in the soluble organic-N fraction. The subtraction of the latter from total-N to yield protein-N by difference lowered the actual proteinaceous values from 2.0% to 25.0% on account of the inclusion of proteose-N in the soluble organic-N fractions. If this correction be taken into consideration, many of observed chlorophyll/protein ratios approach the theoretical value.

Studies of protein structure by ASTBURY (**1, 2**) indicate that the fibrous proteins, have as the unit of structure a polypeptide grid whose normal equilibrium configuration is not flat, but buckled, that is, the main chains are thrown into a series of intramolecular folds lying in planes transverse to the cross-linkage of the grid, and its basis of the long-range elasticity is the capacity to unfold and refold. Moreover, additional evidence pointing to a simple system of close packing of the side chains, the polars on one side of the main chain and the non-polars on the other, suggests that such close packing results in the formation of protein laminae which may appear alone or sandwiched with accessory groupings, such as lipoid laminae. SCHULMAN (**23**) has shown that, in lipoproteins, the pH of the solution with values above or below the isoelectric point of the protein may increase or decrease the the relative degree of association or combination of lipoids with proteins.

In *A. comosus* the pH and titrable acidity values of the sap from different leaf sections vary considerably (**26, 27, 29, 31**). Gradients of titrable acidity and pH of the various leaf sections, influenced by diurnal changes and rates of respiratory activity (**33**), increased in the former but decreased in the latter from the basal to the terminal sections during the hours of darkness or low light, while at times of full sunlight, when photosynthetic activity was at maximum, they either disappeared or became slightly reversed. Thus the different conditions provided in the cell by the constantly changing acidity or pH may increase or decrease the degree of association between chloroplastic proteins and chlorophyll. Hence, the divergence between observed and theoretical chlorophyll/protein ratios may be explained by the effects of pH on the chlorophyll combining power of the chloroplastic proteins of different leaf sections.

Summary

There was positive correlation between chlorophyll and protein in the chlorophyllous regions of plants supplied with adequate amounts of nutrients and seasonal light.

Insufficiency of iron, light, and nitrogen decreased considerably the amounts of chlorophyll and protein in the chlorophyllous regions of leaves.

Potassium in the nutrient solutions supplied in small or high amounts had no measurable influence on the relations of chlorophyll to protein.

Ratios of chlorophyll to protein in the distal relatively mature leaf sections, where both substances reached maximal content, approached the theoretical value of 0.055 of Hanson, but in the proximal less mature sections chlorophyll/protein ratios indicated greater amounts of chlorophyll in proportion to protein.

Protein-N/soluble organic-N ratios were approximately 1:1 in the basal (no. 1) non-chlorophyllous sections and 3:1 in the chlorophyllous (no. 4) sections, indicating that protein-N rather than soluble organic-N is directly related to chlorophyll.

These findings suggest that chlorosis, excluding variegation, in plants is a state of incompatible chlorophyll and protein relations in the chloroplast due to deficiencies of substances essential for the synthesis of chlorophyll or proteins, or to conditions (high Mn) inhibitory for the synthesis of such substances or for the proper operation of the chlorophyll-protein system. This state may be restored to normalcy either by the elimination of the deficiencies or the adverse conditions.

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RADIOACTIVE PHOSPHORUS PROCEDURES AS APPLIED TO SOIL AND PLANT RESEARCH[1]

C. L. COMAR AND J. R. NELLER

The importance of phosphorus in soil–plant relationships is well recognized, as evidenced by the magnitude of the phosphate fertilizer industry in the United States. Knowledge is incomplete, however, regarding the behavior of phosphorus in the soil, its availability for plant uptake, and the extent to which it is affected by the numerous ever-present variables. The isotopic tracer technique, in addition to sensitivity, simplicity, and rapidity, possesses a fundamental advantage over the standard chemical methods in that phosphorus added to a system can be distinguished from that already present.

In 1940 BALLARD and DEAN (3), who reported on the use of radioactive phosphorus in soil studies, used an electroscope for detection purposes and made measurements on soil, plant material ash, and on intact plants. In 1941 BALLARD and DEAN (4) described the application of these procedures in biological measurements of the retention of applied phosphorus by soils; they obtained close agreement between phosphorus fixation when determined by the standard water equilibrium system method, and when determined by the absorption by 19-day-old tomato plants of radioactive phosphorus. They were unable to obtain correlation between the standard method and the use of radioactive phosphorus in the chemical estimation of phosphorus fixation. No data as to the sensitivity of the detection procedures used or the specific activities of the original solutions were presented. The use of radioactive phosphorus in short-term plant physiological studies has been reported by several workers (1, 5, 7, 8).

This technique has not yet been extensively used, principally because of an inadequate source of radioactive materials and the lack of detection equipment during the war years. With cyclotron output now available for peacetime research, however, and with the reasonable expectation that atomic energy plants will soon be used for the production of radioactive isotopes, the main obstacle will be removed (see Science, June 14, 1946). Experience has indicated that field studies are economically feasible in addition to the laboratory and greenhouse scale of experimentation.

It is the purpose of this paper to describe advantageous procedures for the use of radioactive phosphorus in soil and plant studies and to present results with a series of Florida soils. Data are given on the sensitivity of the method, since this factor in consideration with an estimate of the expected

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uptake will determine the practical maximum time limit of the experiment and the activities which must be used.

Materials and method

Radioactive material was supplied by the Massachusetts Institute of Technology, Radioactivity Center, and was prepared by bombardment of phosphorus with deuterons. It was obtained as a purified solution containing 4.2 mg. phosphorus in the form of H_3PO_4, with a half-life of 14.3 days and an initial specific activity of 0.95 millicuries per mg. Using the 15-ml. cell described below, the measurements were sensitive to 0.00015 micrograms phosphorus at 12 days after preparation.

The activity was measured with a Geiger-Mueller counter apparatus (No. 3 combination purchased from Herbach and Rademan, Inc., Philadelphia, Pa.). A dipping type counter tube was employed constructed similarly to that described by BALE, HAVEN, and LEFEVRE (2) (purchased from Distillation Products, Inc., Rochester, N. Y.). BALE et al. described a special mount for holding the counter tube; they used a rack and pinion device for supporting and adjusting the glass cell which contained the solution to be measured. Satisfactory results were obtained, however, by holding the counter tube with an ordinary clamp and ring-stand and by supporting the cell with a pivoted beaker shelf which could be fixed at the desired height and swung aside for removal of the cell (fig. 1). The sensitive portion of the counter tube is a cylinder about 51 mm. in length and 11 mm. in diameter. For maximum sensitivity, a test tube of 15 mm. inside diameter and about 60 mm. in length should be used with 2 ml. of the unknown solution. However, when the samples contain sufficient activity it is more convenient to use larger cells; in the work reported here 15 ml. of solution were used in cells of 22.4 mm. inside diameter and 63 mm. length. The sensitivity was decreased about fourfold by using the 15-ml. cells instead of the 2-ml. cells. For the actual measurement the cell was supported in paraffin contained in a 400-ml. beaker; the cell was held firmly, but it was removable for interchange of samples. No significant differences were observed when the cell was centered with the counter tube merely by inspection or when different cells of the same dimensions were used. The same paraffin-containing beaker should be used for any given series of measurements, since it is difficult to get any two beakers to support the cells at exactly the same height. With the counter tube and beaker shelf fixed in position, and using a given volume of solution in cells of identical dimensions in the same paraffin-containing beaker, reproducible results were obtained.

The background, due primarily to cosmic radiation, averaged 12 counts per minute for the set-up as described and was usually measured twice during the day. Each solution was measured for 10 minutes or longer, depending on the activity contained, and net counts of 4 per minute or less were not considered significant.

The absolute radioactive phosphorus content was obtained by the use of

standard solutions, prepared by using known amounts of the original radioactive phosphorus solution, carried through the regular procedure for the given experiment. The calibration curve was then constructed by plotting the weight of phosphorus against the count per minute. Instead of correcting for decay by calculation back to some zero time, a calibration curve was run during the middle of each working day for use with the samples measured on that day. Actually about 3 points were sufficient to determine the curve for routine work, and the position of the curve from day to day indicated that the instrument was operating properly.
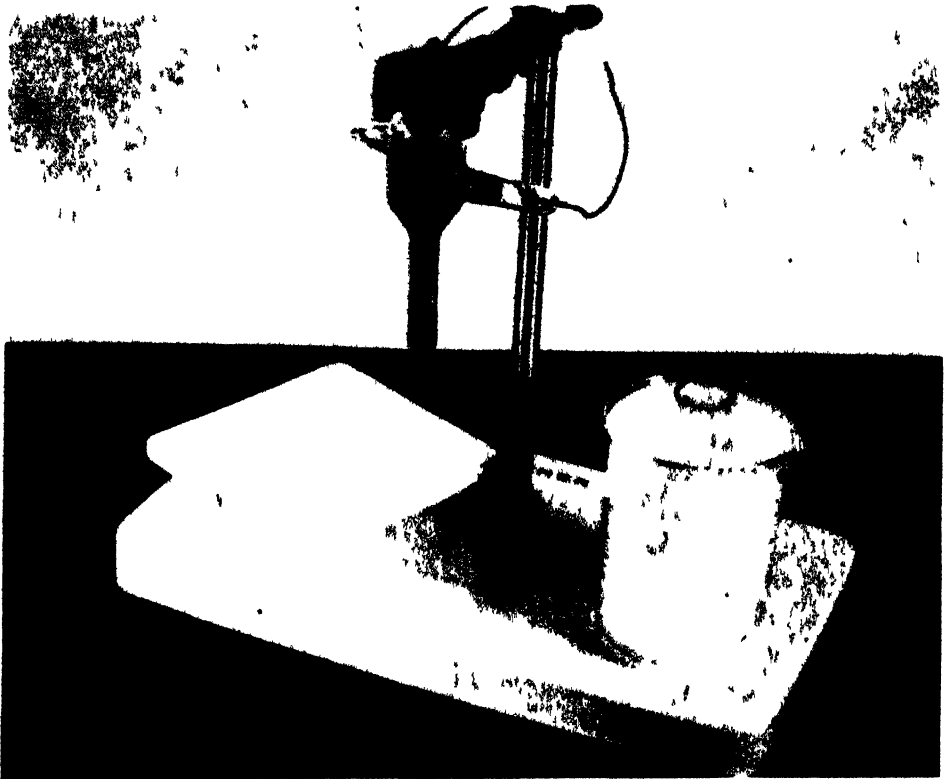

FIG. 1. Apparatus for measurement of radioactivity in solutions. Geiger Mueller counter tube, swinging beaker shelf, and 15 ml. cell supported in 400 ml. paraffin containing beaker.

The amount of radioactive material needed for a given experiment is fixed by (a) the sensitivity of the detection procedure at the time the measurements are to be made and (b) the amount of the isotope which will have accumulated in the sample. If the latter can be estimated, then the appropriate dosage can be calculated; the following example will illustrate how this was done for some of the experiments. In the greenhouse and lysimeter-jar studies it was expected that even those plants in soils of high fixing capacity would take up not less than 1% of the added phosphorus. It was decided that the measurements would be made not more than 3 months after the date on which the sensitivity had been determined as 0.00015 micrograms phosphorus; due to radioactive decay the sensitivity after 3 months would be

about 0.01 micrograms. For optimum measurement the sample should contain at least 10 times the minimum activity, and, since 2 samples were to be taken from each jar, it was calculated that an aliquot of the original solution containing about 20 micrograms of phosphorus added to each jar would be in the proper range.

Results and discussion

PHOSPHORUS FIXATION BY SOILS

The degree of phosphorus fixation by soils may be determined satisfactorily by chemical methods of analysis. The advantages in the use of the radioactive-tracer method are (a) simplicity and rapidity, (b) use of minute amounts of added phosphorus and (c) elimination of need to correct for behavior of original phosphorus in the soil.

The general procedure was as follows: an extracting buffer solution of dilute acetic acid and sodium acetate as described by MORGAN (6) was employed. For the equilibrium studies appropriate amounts of radioactive phosphorus in the form of Na_2HPO_4 plus the inert phosphorus as KH_2PO_4 were incorporated in the extracting solution, and 50 ml. of this solution were agitated with 10 gm. of soil in a mechanical shaker for 30 minutes. The mixture was filtered, and the amount of radioactive phosphorus recovered was determined by direct measurement on the filtrate. Some of these solutions were analyzed chemically, and corresponding values for recovery were calculated by correcting for the phosphorus appearing in the filtrate when none was added originally.

In the extraction experiments 10 ml. of solution, containing the active and inert phosphorus, were added to the soil sample, which was then air dried, agitated with 50 ml. extracting solution, and carried through the procedure as described above.

Table I shows the phosphorus recoveries from the various soil types as obtained by the use of the different procedures; the values are the averages of duplicate determinations in all cases. The values from chemical analysis were in fair agreement with those obtained by the isotope technique, and the rating of the soils as to phosphorus fixation was the same by either method. Where the soils were air-dried after the addition of the phosphorus, the recovery was decreased in all cases, especially with the sandy loams and clay types. In general, there was a greater percentage of phosphorus recovered at the 200-p.p.m. level than at 50 p.p.m., particularly with fine sands; however, there was little difference with the Dunbar very fine sandy loam and Coxville clay. At the 400-p.p.m. level the phosphorus recovery was generally greater than at 200 p.p.m. although there appeared to be no simple proportionality between the initial amount added and the percentage recovery.

PHOSPHORUS UPTAKE BY THE PLANT

The value of an extraction procedure is determined by the extent to which the extracting solution removes those forms of phosphorus which are of

TABLE I

RECOVERY OF PHOSPHORUS ADDED TO THE SURFACE HORIZONS OF VARIOUS TYPES OF SOIL,
USING ACETIC ACID–SODIUM ACETATE EXTRACTING SOLUTION

SOIL TYPE	AMOUNT PHOSPHORUS ADDED	PERCENTAGE RECOVERY OF PHOSPHORUS		
		CHEMICAL ANALYSIS OF EQUILIBRIUM SOLUTION	RADIOACTIVE PHOSPHORUS IN EQUILIBRIUM SOLUTION	RADIOACTIVE PHOSPHORUS IN EXTRACTION AIR-DRY SOIL
	p.p.m.	%	%	%
Leon fine sand	50	70	76	52
	200		88	61
	400		83	66
Hernando fine sand	50	54	51	16
	200		68	23
	400		76	25
Portsmouth fine sand	50	39	45	16
	200		59	25
	400		66	31
Norfolk loamy sand	50	43	43	6
	200		61	10
	400		66	11
Dunbar very fine sandy loam	50	20	20	8
	200		22	6
	400		32	6
Amite sandy loam	50	19	17	2
	200		26	3
	400		38	5
Coxville clay	50	12	13	3
	200		13	3
	400		11	4

immediate significance to plant growth. The availability of added soil phosphorus depends upon many factors, particularly the nature of the soil and the type of plant. The problem of correlating the actual utilization of nutrients by plants with the results of soil extraction procedures has been a difficult one. The use of the tracer technique affords a simple method of following the actual plant uptake with physiological amounts of the element, and

TABLE II

RECOVERY IN OAT TOPS OF RADIOACTIVE PHOSPHORUS ADDED TO VARIOUS TYPES OF SOIL

SOIL TYPE	CONTAINER	NUMBER OF CONTAINERS	GROWTH*	ADDED RADIOACTIVE PHOSPHORUS RECOVERED IN OAT TOPS
			gm.†	%
Leon fine sand	Greenhouse jar	6	3.5 ± 0.2	33.0 ± 2.0
Leon fine sand	Lysimeter jar	4	20.2 ± 0.5	32.9 ± 1.3
Hernando fine sand	Greenhouse jar	6	3.9 ± 0.7	16.8 ± 3.5
Amite sandy loam	Lysimeter jar	2	19.6 ± 0.8	13.0 ± 0.4
Coxville clay	Lysimeter jar	2	17.1 ± 1.6	11.3 ± 2.1

* Grams of dry weight of tops.
† The mean value ± the mean deviation.

furnishes a convenient means of comparing the extraction procedure with the biological method under controlled conditions.

In one set of plant experiments glazed greenhouse jars were employed, holding 1000 gm. of soil to which 58 p.p.m. phosphorus as citrate soluble phosphate had been added. About a dozen oat plants were grown in a circle in each jar; when the seedlings were about 2 inches high the appropriate amount of radioactive phosphorus in 10 ml. solution was added to a hole 2½ inches deep punched in the center of the soil area. The jars were watered regularly according to usual greenhouse practice. In other studies 4-gallon glazed lysimeter jars were employed holding 14,000 gm. of soil; the top 4 inches, 7000 gm., had previously been treated to contain 49 p.p.m. phosphorus. About 40 oat plants were grown in each lysimeter jar and the procedure followed as described above.

Radioactive phosphorus was applied February 21 and the plants harvested April 25, 1946. The tops were dried and weighed to give the growth data; samples, usually 2 gm., were ashed with magnesium nitrate according to the usual methods for phosphorus determination. The ash was taken up in hydrochloric acid solution, made to volume, and the activity measured as earlier described.

Table II shows the recovery of radioactive phosphorus in the tops of oats grown on various types of soil. The amount of growth was about the same regardless of the soil type. The percentage recovery from the Leon fine sand was the same, whether the greenhouse or the lysimeter jars were used. With respect to phosphorus recovery the soils ranked in the same order as determined by the chemical and tracer methods; however, the differences were not so great as might have been expected from the values reported in table I. Using the 50-p.p.m. level as the basis of comparison, it was evident that chemical analysis and radioactive phosphorus in equilibrium solution gave the best correlation with plant uptake. With these methods the relative fixing capacities of the Leon fine sand and the Hernando fine sandy loam were in close agreement; this was true also for the Amite sandy loam compared with the Coxville clay. However, a comparison of the Leon and Hernando with the Amite and Coxville showed less differences in fixing capacities when measured by plant uptake than when measured with radioactive phosphorus in equilibrium solution.

The Leon fine sand is of particular interest since it is widespread throughout Florida and has usually been found deficient in phosphorus. About 33% of the added phosphorus was recovered in the oats grown under what might be considered as intensive cropping. Under the experimental conditions reported here it would have been necessary to use only 0.6 micrograms of the phosphorus preparation per jar for adequate measurements, which corresponds to an initial activity of about 0.6 microcuries. This means that field work, leaching studies, or the growing of plant material containing radioactive phosphorus to be fed to animals for metabolism investigations will not be economically prohibitive.

Summary

Convenient procedures for the use of radioactive phosphorus in soil-plant research are described. With a sample having an initial specific activity of 0.95 millicuries per mg. the measurements were sensitive to 0.00015 micrograms phosphorus at 12 days after preparation.

A series of Florida soils showed the same ranking with respect to phosphorus fixing capacity as determined by (a) chemical analysis of equilibrium solution (b) radioactive phosphorus in equilibrium solution (c) radioactive phosphorus in extraction of air dry soil and (d) uptake of radioactive phosphorus by oat plants grown for 63 days.

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RELATIONS BETWEEN CARBOHYDRATE ACCUMULATION AND RESISTANCE OF COTTON PLANTS TO PHYMATOTRICHUM ROOT ROT IN DRY SUMMERS[1]

FRANK M. EATON, E. W. LYLE, AND D. R. ERGLE

(WITH TWO FIGURES)

As observed by several investigators (3, 4, 9, 11, 12, 13, 14), phymatotrichum root rot is notably less severe in dry than in moist summers. Increases have likewise been noted in the numbers of plants dying from root rot after rains and irrigations. The question has not been raised before, however, as to whether the low root-rot mortality of cotton plants in dry seasons is due to moisture conditions unfavorable to the spread of the fungus, *Phymatotrichum omnivorum* (Shear) Duggar, through the soil, or to an associated change in the disease resistance of the plant.

Increased resistance of cotton plants to phymatotrichum root rot has been shown elsewhere by EATON and RIGLER (2) to follow increases in the carbohydrate concentrations in the root bark. In that investigation carbohydrate levels were altered by such means as defruiting, partial defoliation, and adjustments in nitrogen supply; the effects of drought on carbohydrate levels and upon root rot were not considered.

The data developed in this paper deal with (1) the effect of drought on the accumulation of carbohydrates and nitrogen in the root bark of cotton plants growing in dry and irrigated field plots, (2) with the effect of drought, in the same experiments, on the severity of phymatotrichum root rot, and (3), by means of laboratory measurements, with the ability of the fungus to grow from a moist substrate into soils with high moisture tensions.

Methods

The concentrations of sugars were measured by the WILDMAN and HANSEN semi-micro procedure (15), starch was hydrolyzed with diastase, and hemicellulose with hydrochloric acid. These methods have been reported in greater detail elsewhere (1). The procedures followed in the field experiments and in the laboratory measurement of fungus growth are dealt with in conjunction with those results.

Results

CARBOHYDRATE ACCUMULATION AND ROOT ROT IN DRY AND IRRIGATED PLOTS

Field experiments were conducted at the Blackland Substation, Temple, Texas, in 1942 and again in 1944. The experiments included dry and irrigated plots on Houston Black clay in a field that had shown much root rot in previous years.

The 1942 experiment was looked upon as exploratory and was laid out in only two blocks, each with four plots. The four treatments were as follows: (1) irrigated, (2) irrigated and fertilized, (3) dry, and (4) dry and fertilized. Five acre-inches of water were applied to the irrigated plots on July 10 and again on August 10. The rainfall was negligible between the middle of June and August 12, but on the latter date there was a rain of 0.48 inches, and on August 16 there was a rain of 1.56 inches. Rains of 0.61, 0.13, 0.52, and 0.11 inches fell on August 19, 24, 30, and 31, respectively. During the first ten days of September rainfall amounted to 6.35 inches. The fertilized plots received 800 pounds per acre of 9–6–3 commercial fertilizer placed beneath the drill rows at time of planting (May 4).

The plants for root-bark samples were collected between 10:00 A.M. and

TABLE I

MEANS OF CARBOHYDRATES, TOTAL NITROGEN, AND MOISTURE CONCENTRATIONS IN ROOT BARK (ONE DATE) AND PLANTS KILLED BY ROOT ROT (ONE DATE) IN UNFERTILIZED WET AND DRY PLOTS IN 1942*

ITEM	WET		DRY	
	UNFERTILIZED	FERTILIZED	UNFERTILIZED	FERTILIZED
	%	%	%	%
Root-bark constituents:				
Hexoses	0.56	0.68	1.11	1.05
Sucrose	1.02	1.07	1.53	1.98
Starch	0.84	0.86	1.03	1.16
Total CHO	2.42	2.61	3.41	4.20
Hemicellulose	9.12	9.63	9.79	10.45
Total nitrogen	0.130	0.152	0.136	0.201
Moisture	79.0	78.0	75.4	75.4
	gm.	gm.	gm.	gm.
Weight tap roots	17.1	21.7	11.2	12.2
	mean %	mean %	mean %	mean %
Plants killed by root rot: on Oct. 1	50	52	27	21

* Analytical data expressed as percentage of fresh weight.

11:00 A.M. on the morning of August 17 after irrigation of the irrigated plots on August 10 and after rains of 0.48 inches and 1.56 inches on August 12 and 16, respectively. At no time did the plants in the dry plots show any marked degree of wilting, but it may be noted that the two irrigations resulted in a large increase over the dry plots in the weight of the tap roots (table I). Each of the 16 samples for carbohydrate analysis was a composite of the root bark from 15 uninfected plants selected at random in the upper and lower halves, respectively, of each of the 8 plots.

Although samples were collected following a day of rain, the concentrations of hexoses, sucrose, and starch were all higher in the dry than in the irrigated plots. Drought resulted in an increase of 51% in the sum of these carbohydrates (table II). Fertilization also increased carbohydrate

concentrations, but it is probable that part of the increase is attributable to differences in moisture supply; *i.e.*, with the same amount of rain and irrigation, the fertilized plants were larger as shown by the 18% increase in weight of tap roots.   The slightly higher concentrations of hemicellulose found in the plants from the dry plots disappear if the data be computed to a residual dry weight basis.   There were twice as many plants killed by root rot in the irrigated as in the dry plots on October 1 (table I) and similar differences were found also on September 1 and 15.

The 1944 experiment was undertaken on a more comprehensive scale than the one in 1942, and the summer was more favorable for it.   There were 16.75 inches of rain between May 1 and June 12, but thereafter there were no rains of consequence until the period of August 27 to 30 when 1.91

TABLE II

PERCENTAGE CHANGE AS A RESULT OF DROUGHT AND MINERAL FERTILIZER ON ROOT-BARK CONSTITUENTS AND PLANTS KILLED BY ROOT ROT IN 1942—FACTORIAL SUMMARY

ITEM	DROUGHT*	FERTILIZER†
	%	%
Root-bark constituents:		
Hexose	74§	4
Sucrose	68§	28‡
Starch	29‡	11
Total CHO	51§	17‡
Hemicellulose	7	6
Total nitrogen	19§	3‡
Moisture	− 3§	− 1‡
Weight	−40§	18§
Plants killed	−53‡	− 5

* Determined by the difference between the means of wet and dry treatment results.
† Determined by the difference between the means of the unfertilized and fertilized treatment results.
‡ Statistically significant by odds of 19 to 1.
§ Statistically significant by odds of 99 to 1.

inches fell.   On September 6 and 7 there was another rain of 1.68 inches. Starting on July 14 the wet plots were irrigated with one acre-inch of water weekly.   Fertilization consisted of 6.4 tons of oat straw (air-dry basis) plus 100 pounds of inorganic nitrogen per acre.   These materials were disked into the soil prior to planting on May 13.   The four treatments: (1) irrigated, (2) irrigated plus manure, (3) dry, and (4) dry plus manure, were randomized in each of ten blocks.   Dead-plant counts were made at intervals of about 15 days starting in mid-July.   The means of the percentages of dead plants on July 31, August 14, September 1 and September 15 are shown (table III).   On September 15 there were, respectively, 67% and 76% of the plants dead in the unfertilized and manured irrigated plots, and 13% and 10% in the corresponding dry plots.   The soil-moisture samples were taken at a depth of 6 to 8 inches on the day before the irrigations of July 27, August 3, 10, 18, and 24; the root-bark samples were collected between 10:00 A.M. and 11:00 A.M. on each of four dates during the fruiting

period (fig. 1). Each collection was in triplicate for each treatment, with one uninfected root from each of ten plots making a single sample.

The total-carbohydrate concentrations as found on each of the four dates in 1944 (fig. 1) are summarized as separate constituents (table III). Consistent with the earlier results, the hexoses, sucrose, and starch concentrations were higher in the dry than in the irrigated blocks. The greatest effect of drought was found in starch concentrations. Little effect of manuring is reflected in the hexoses or sucrose sugars, but manuring resulted in large decreases in starch, both in the wet and dry plots.

Drought greatly increased the concentrations of total nitrogen in the root bark (table IV). Manuring, however, had only minor effects on nitrogen

TABLE III

MEANS OF CARBOHYDRATES, TOTAL NITROGEN, AND MOISTURE CONCENTRATIONS IN
ROOT BARK (FOUR DATES) AND PLANTS KILLED BY ROOT ROT (FOUR DATES)
IN UNFERTILIZED AND MANURED WET AND DRY PLOTS IN 1944*

ITEM	WET		DRY	
	UNFERTILIZED	MANURED	UNFERTILIZED	MANURED
	%	%	%	%
Root-bark constituents:				
Hexose	1.00	1.02	1.18	1.21
Sucrose	1.69	1.72	2.13	2.07
Starch	1.27	0.95	2.46	1.79
Total CHO	3.96	3.69	5.77	5.08
Total nitrogen	0.242	0.268	0.365	0.359
Moisture	74.6	75.4	70.1	71.0
	gm.	gm.	gm.	gm.
Weight per bark	11.8	13.3	8.4	9.0
	mean %	mean %	mean %	mean %
Plants killed by root rot	38	42	10	6

* Analytical data expressed as percentage of fresh weight.

levels in the experiment, and the responses were greater in the dry than in the irrigated plots.

The relations found between the severity of root rot and carbohydrate concentrations in the root bark are shown (fig. 1) by graphing carbohydrates and root rot, the latter being expressed as the percentages of living plants which died during the successive periods of the experiment. Root rot first makes its appearance in cotton fields in the summer as scattered dead or wilted plants. As these centers of infection, and new ones, enlarge concentrically, it is the plants on the peripheries that are subject to attack. As the peripheries become longer, increasing numbers of live plants are in positions to be attacked, but as the margins of adjacent dead areas come together, the trend in the number of plants subject to attack may be reversed.

Irrespective of whether the spread of the fungus from plant to plant is primarily along overlapping roots or by the growth outward of the fungus

through the soil, cotton roots provide the organic substrate, and the spread
of the fungus is largely limited by root spread. The rate of spread of the
disease is thus dependent, among other conditions, upon the availability of
the organic substrate provided by the cotton roots and the suitability of this
root material for the fungus. The measurement of this suitability (disease
resistance) can be made fairly direct in the case of plants that are growing
in pots where the days from inoculation to death can be measured; measure-
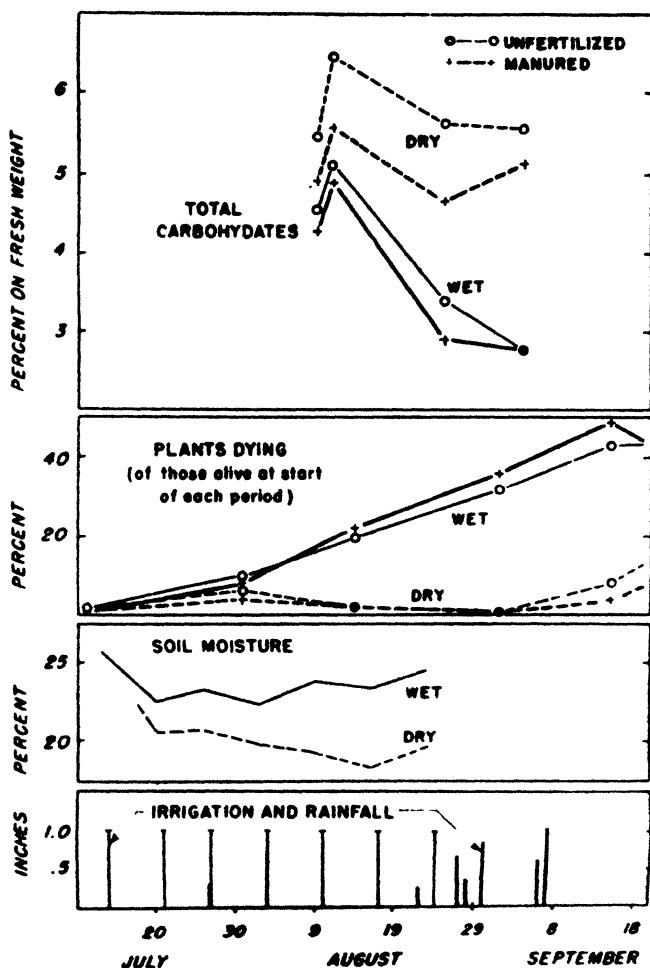
Fig. 1. Carbohydrate levels, severity of phymatotrichum root rot, soil moisture,
rainfall, and irrigation during the 1944 experiment.

ment cannot be so direct in the field, however, for there are no means for
determining the time lapse between infection and death.

Notwithstanding these difficulties of measurement, a general inverse
relation is shown (fig. 1) between differences in carbohydrate levels in the
plants and differences in root rot between (1) the unfertilized irrigated and
manured irrigated plots and (2) between these two treatments and the two
dry treatments considered collectively. There was as little root rot in the
dry plots with manure as in those without manure, but there were much

lower carbohydrate levels in the former. The effects of the manure can be considered in this connection.

Various investigators have found manuring to be one of the most effective means of controlling root rot. As reported by KING and LOOMIS (6) a 10-ton application of manure to irrigated plots in Arizona resulted in much lower percentages of death until mid-August, but later the rate of spread was as great in the manured as in the check plots; presumably by this time the decomposition of the manure had proceeded to the point that it no longer supported competitive or antibiotic organisms. In the present experiments the data show no evidence that the 6.5 tons of manure provided antibiotic protection against root rot in the wet plots, but this was not necessarily the case in the dry plots. The manure decomposed rapidly in the wet plots, but a good bit of it was still in evidence in the dry plots in late September.

TABLE IV

PERCENTAGE CHANGE AS A RESULT OF DROUGHT AND MANURE ON ROOT-BARK
CONSTITUENTS AND ROOT ROT IN 1944—FACTORIAL SUMMARY

ITEM	DROUGHT*	MANURE†
	%	%
Root-bark constituents:		
Hexose	18.8§	2.8
Sucrose	23.5‡	0.0
Starch	91.0§	− 26.3§
Total CHO	41.9§	− 9.9
Total nitrogen	42.0§	3.2
Moisture	− 6.1§	1.4§
Weight	− 31.0§	10.8§
Root rot	− 80.0§	0.0

    * Determined by the difference between the means of wet and dry treatment results.
    † Determined by the difference between the means of unfertilized and manured treatment results.
    ‡ Statistically significant by odds of 19 to 1.
    § Statistically significant by odds of 99 to 1.

In other words the lower root-rot mortality in the dry-manured plots than in the dry plots without manure—relative to carbohydrate levels—could have been due entirely to the antibiotic effects of the manure.

There remain for consideration relations between the rates of plant death and variations in carbohydrate levels as found at the four sampling dates. The carbohydrate levels found on August 9 and 11 were higher than subsequently and there was a correspondingly lower mortality rate in the irrigated plots in early August. It is doubtful, however, that any significance can be attached to this relationship. The four sets of samples were sufficient to establish the relative levels of carbohydrates under the four treatments, but the four collection dates do not necessarily reflect variations in carbohydrate levels between sample collections. Furthermore, the method employed for representing resistance to root rot by rate of plant deaths cannot be expected to reflect accurately day-to-day changes in resistance.

GROWTH OF FUNGUS INTO SOILS OF VARIED MOISTURE CONTENT

Concurrently with the establishment of *Phymatotrichum omnivorum* on and in a cotton root, hyphal wefts grow forward along the root, etching the epidermis some distance in advance of actual invasion. The root-rot fungus probably makes its most rapid advance from plant to plant along roots, but it is also certain that there is a substantial outward growth through the soil that results in the establishment of the fungus in nearby roots. Irrespective of the main avenue of advance, it is reasonable to expect that the growth of
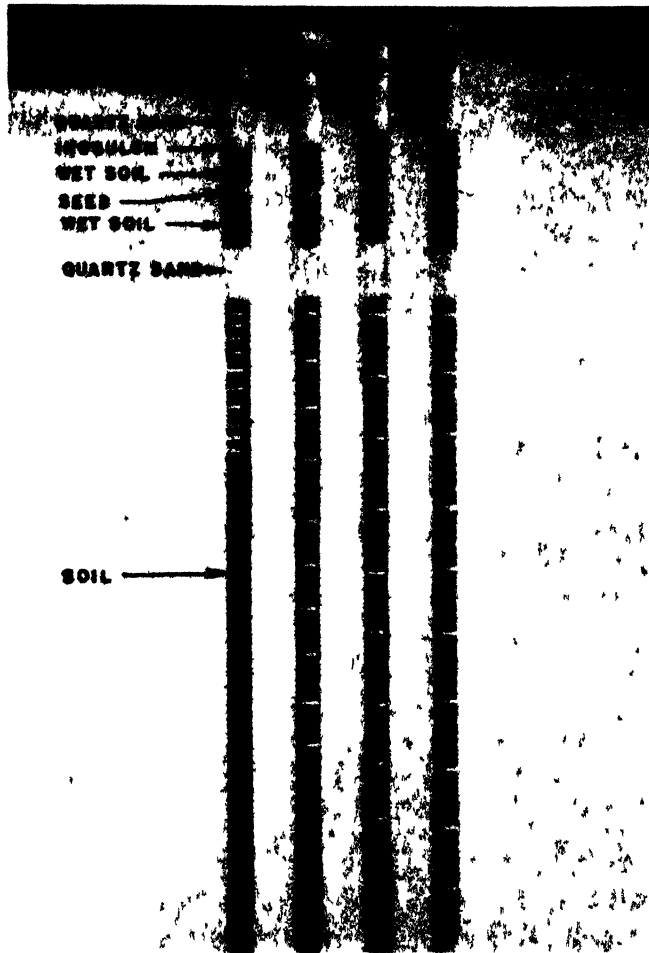
FIG. 2. Daily growth rate of *P. omnivorum* from a moist substrate (across a quartz-sand bridge) into soils of varied moisture content.

this fungus, unless it be effectively protected against evaporation by a coating of suberin-like materials or by a physico-chemical mechanism for water retention, would be greatly influenced in its growth by the tension of the soil moisture. If in dry soils the root-tissue substrate provides most or all of the moisture required for growth, and water loss from the mycelium to the soil occurs very freely, then water movement from the tissue substrate through the strands and hyphae would have to proceed with great rapidity.

In the light of the cellular structure of this fungus, it is difficult to understand how a very rapid movement of water could occur. In any event a need is indicated for evidence of the ability of the fungus to grow from a moist substrate into soils with high moisture tensions.

An experiment was undertaken for the purpose of measuring the rate of growth of the fungus from a moist substrate into soils of varied moisture content. Both the means adopted for the measurements and representative results are illustrated (fig. 2). The importance of the information relative to one of the major considerations of this paper is fairly obvious. If the growth of this fungus be sharply restricted as soils become dry, even though provided with a source of moisture in root tissues, there could be little progress of the disease. This would be the case whether or not the plant, through the consequences of its own reactions to low soil moisture, had developed a high degree of resistance.

The Houston Black clay employed for the experiment had a moisture equivalent of 30.9%. Aliquots of this soil were adjusted to four moisture percentages by water additions and sieving. Each of the soils was slowly added, with a substantial loss of moisture, to glass tubes 15 mm. in diameter and approximately 60 cm. long to a depth of 43 cm., while the bottoms of the tubes were tapped sharply on the surface of a table. On top of the soil there were added in sequence: 4 cm. of coarse quartz sand (to prevent capillary movement), 3 cm. of dry soil, 25 moistened sorghum seeds, 3 cm. of dry soil, 10 sorghum seeds, and lastly 4.5 ml. of water and a cotton plug. After standing upright overnight the tubes were autoclaved for one hour on two successive days and inoculum then added on top of the upper seeds. The tubes were finally filled to the plugs with sterile sand. Neither the sand nor the cotton plugs provided adequate protection against evaporation; at the end of the experiment the outer half of the moist soil above the sand bridge was found to be drier than the inner half. Some vapor movement from the wet soil into the lower soil was likewise indicated. The approximate initial moisture percentages for the soil in place in the tubes are the rounded values found for the 7-day to 9-day segments at the end of the growth period (table V). There were four tubes for each of the four moisture levels.

Sterile methods were necessary to prevent the decomposition of the sorghum-seed substrate by organisms other than *P. omnivorum*. The tubes were kept on their sides in a transfer room at 28° C. during incubation and while the daily growth measurements were being recorded. Measurements were referenced to a crayon mark that located the position of the fungus in the soil on the first morning after it had grown through the sand bridge. This date was not the same for all tubes.

There was little difference in the rate of growth of the fungus into soils A and B with respectively 27% and 23% of moisture (table V). The growth rate in the C tubes, which contained initially 20% moisture was 70% of that in the A and B tubes. Growth in the D tubes, with 15% initial moisture, was 23% as great as in the A and B tubes.

The "first permanent wilting point" and the "ultimate wilting point" as determined by the method of FURR and REEVES (5) were for this soil 22.2 and 18.1, respectively. Knowing these values it is possible to orient roughly the soil moisture percentages of the soil used in the various tubes in terms of soil moisture tensions. As determined by the pressure-membrane method, RICHARDS and WEAVER (10) found the tensions for 24 of the Furr-and-Weaver soils at "first permanent wilting" to range from 5 to 13 atmospheres with 14 of these soils in the 7 to 9 atmosphere range. At "ultimate permanent wilting" 17 out of 24 of the soils fell within the 20 to 30 atmosphere range.

It is evident that the outward growth of the fungus from a moist substrate was not affected by soil-moisture tensions (the B tubes with 23% moisture) approaching, if not exceeding, 5 atmospheres. Growth was re-

TABLE V

GROWTH OF *Phymatotrichum omnivorum* INTO SOILS OF VARIED MOISTURE CONTENT
WHEN SUPPORTED BY SORGHUM SEED IN A MOIST-SOIL SUBSTRATE

TUBES	INITIAL MOISTURE CONTENT OF SOILS IN FILLED TUBE	MEAN GROWTH OF FUNGUS			SOIL MOISTURE		
		SECTION 0–3 DAY	SECTION 4–6 DAY	SECTION 7–9 DAY	SECTION 0–3 DAY	SECTION 4–6 DAY	SECTION 7–9 DAY
	%	mm./day	mm./day	mm./day	%	%	%
A	27	44	44	42	28.6	27.6	27.0
B	23	45	41	40	25.9	23.2	23.0
C	20	31	32	30	20.7	19.8	20.3
D	15	9	10	10	18.4	16.3	15.0

tarded only 30% by tensions probably in the range of 10 to 20 atmospheres in the C tubes that had 20% moisture. The fungus was able to make a substantial growth into a soil with moisture tensions that may have been well in excess of 30 atmospheres—the D tubes with 15% moisture.

The experiment provides no explanation of the ability of *P. omnivorum* to retain or move its water and grow into the drier soils. This ability is of special interest because of the fact that the new hyphae had diameters in the order of 0.01 mm. The single hyphae advanced ahead of strand formation by about one day, and the largest strands and occasional sclerotia developed three or four days later.

The foregoing results are largely in accord with earlier measurements by ROGERS (11) who placed soil (of the same type and collected from near where the present soil was obtained) in jars and inserted pieces of infected cotton roots. A substantial growth of the fungus occurred in soil with 15% moisture, and 25% moisture appeared to him to be near the optimum.

The fact that *P. omnivorum* is found to possess a remarkable ability to grow from a moist substrate into soils with high moisture tensions bears on the observed low incidence of the disease in the dry plots. A greater death rate of plants in these plots should have occurred if the direct effects of low

soil moisture on the growth of the fungus had been the only factor retarding its spread—either along roots or from the roots of one plant to those of another. In other words, a plant resistance factor is indicated as having been responsible for a large part of the low root-rot mortality in the unfertilized dry plots of the experiments and, more broadly, in dry years generally.

Discussion

In the investigation (2) that preceded this one it was shown that marked changes occurred in the numbers of root-surface saprophytes as carbohydrate levels in the root bark were increased; at the higher carbohydrate levels the numbers of some types of organisms were increased and others were decreased. Inasmuch as *P. omnivorum* makes an excellent growth on high carbohydrate substrates under sterile conditions, it was concluded that the increased resistance was probably the result of the competition or increased antibiotic activities of one or more of these root-surface saprophytes and the parasitic activity of this root-rot fungus was demonstrated by means of experiments with maize. After inoculation with *P. omnivorum*, maize plants growing on sterile sand-bentonite substrates were rapidly attacked and killed, whereas the roots of maize plants on otherwise similar but nonsterile substrates remained healthy even though paralleled for long distances by strands of the fungus.

Data bearing directly on the effects of drought on the root-surface microfloras of cotton plants are not available, but MITCHELL, HOOTON, and CLARK (7) have presented results which show that major changes occurred in the numbers of various root-surface organisms with the advance of a summer at Greenville, Texas, and further, that some of these variations were obviously due to factors other than the aging of the cotton plants.

The present data extended to the effects of drought, the earlier showing (2) that increased resistance to phymatotrichum root rot results when carbohydrate levels are increased by such diverse means as withholding nitrogen and defruiting and debranching both with and without increased light intensities. The possibility remains that the changes effected in carbohydrate levels by various means altered or accompanied changes in some other constituent of the root bark which was more immediately responsible, either directly or *via* altered antibiotic activity, for changes in resistance. It was shown in previous work, however, that the level of total nitrogen in the root bark apparently is not involved.

The equilibriums between sugars and starch are rarely uniform either throughout the daily cycle or from day to day. Neither in this paper nor in the previous one (2) has any great effort been made to conclude which of the determined carbohydrate components might bear the most direct relation to changes in resistance. In a field experiment somewhat higher correlation between the number of dead plants and the sum of the sugars was obtained than between resistance and total carbohydrates. Tables II

and VII of the previous paper (**2**) in which relations between the carbohydrates and root-surface saprophytes of healthy roots were considered, present evidence that sugars correlated better than total carbohydrates with the changes in the numbers of some, but not all, of the root-surface saprophytes. Prior to actual invasion of the fungus into the root tissue it would seem that there might be a more direct effect of sugars on the root-surface microflora than of total carbohydrates by reason of possible equilibriums between interior and exterior sugar concentrations. During decomposition, however, after invasion has occurred, the hydrolysis of less labile carbohydrate and other tissue components is actively in progress. The growth of *P. omnivorum* is primarily dependent upon the substrate provided by this decomposing mass of tissue, and during the decomposition many constitutents contribute to the growth of the fungus and affect also the nature and activities of the competing saprophytic microflora.

Summary

Phymatotrichum root rot of cotton has been observed for many years to be much less severe in dry than in moist summers. This investigation was directed toward the question of whether low plant mortalities during periods of drought represent an inability of the fungus, *P. omnivorum*, to grow outward from the root substrate to infect neighboring plants or whether the disease resistance of the plants is altered by drought.

Laboratory measurements are presented which show that *P. omnivorum* when supplied with a moist substrate makes almost as rapid a growth outward into soils with moisture percentages within the wilting range as into soils with higher moisture percentages.

During two summers it was found that root rot was far more severe and carbohydrate concentrations in the root bark of cotton plants were much lower in irrigated than in dry plots. The inverse parallelism found between the severity of root rot and carbohydrate concentrations, as influenced by moisture supply, is in agreement with results of previous investigations in which carbohydrate levels were altered by such diverse means as limiting the nitrogen supply and debranching and defruiting. The data are discussed briefly in relation to changes found previously in the numbers of various root-surface saprophytes when carbohydrate levels were increased, and the possible bearing of stimulated antibiotic activity on the results obtained.

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BRIEF PAPERS

INVERSE CORRELATION BETWEEN RUBBER HYDROCARBONS AND INSOLUBLES IN TOTAL SOLIDS OF LATEX FROM *HEVEA BRASILIENSIS*

W. S. STEWART AND R. W. HUMMER[1]

(WITH ONE FIGURE)

Recent studies of the total solids in latex from the rubber-bearing plant *Cryptostegia grandiflora* indicated that a linear inverse correlation existed between the percentages of rubber hydrocarbons and a crystalline substance isolated from the insolubles (1). This stimulated a similar investigation into the relationship between rubber hydrocarbons and insolubles in the total solids of latex from *Hevea brasiliensis*.

Accordingly, thirty-four samples of latex from *H. brasiliensis* were collected and analyzed for rubber hydrocarbons and insolubles. In these samples a wide distribution of rubber hydrocarbons from low to high percentages was intentionally sought by bleeding petioles and stems of young seedlings as well as trunk-tapping mature trees in production. Except for six collections, the latex was air-dried on a glass sheet immediately upon exudation. When it was dry enough to allow handling, it was removed from the sheet and further dried to constant weight at 60° C. The dried latex, called "latex total solids," formed the basis on which percentage composition was calculated. The six latex samples not dried on a sheet of glass immediately after exudation were "rubber biscuits" formed in commercial latex collecting cups as remnants of dried latex from the previous tapping. All latex samples were collected from April, 1944, to March, 1945, from two widely separated locations in Mexico and from two locations in Costa Rica. Because of the low latex yields from petioles and seedlings these samples were composite collections of from five to forty individuals. The trunk latex samples from producing trees of seedling origin were analyzed on an individual trees basis. This allowed for more variation between samples than if they had been composited. The considerable diversity represented by the collections is shown (table 1).

Duplicate analyses of the total solids for rubber hydrocarbons were made by a bromination technique while insolubles were determined gravimetrically (1). By these methods, rubber hydrocarbons and insolubles were thus both directly determined. This is an important point since these two fractions together constitute a large percentage of the total solids. If, therefore, either of them was determined indirectly by difference, an ap-

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TABLE I

ANALYSES OF LATEX TOTAL SOLIDS FROM *Hevea brasiliensis*. UNLESS INDICATED OTHERWISE, LATEX WAS COLLECTED BY TRUNK TAPPING. TRUNK LATEX SAMPLES ARE FROM INDIVIDUAL TREES; ALL OTHER SAMPLES ARE COMPOSITE YIELDS OF FROM 5 TO 40 INDIVIDUALS

DATE	LATEX SOURCE AND TREE AGE	LOCATION	LATEX ANALYSES		
			RUBBER HYDROCARBON	INSOLUBLES	UNDETERMINED (DIFFERENCE)
			%	%	%
Mar. '45	Leaf petioles	El Palmar, V. C., Mexico	47.3	36.7	16.0
"	½-¾ inch dia. green lat. br.	" "	49.3	39.4	11.3
May '44	Mature petioles, 1½-yr. seedling	Gomez Farias, Tps., Mex.	49.4	34.3	16.3
Mar. '45	½-¾ in. gr. side branches	El Palmar, V. C., Mexico	52.3	34.0	13.7
"	Leaf petioles on 2-yr. tree	" "	54.0	33.0	13.0
July '44	Stems, seedlings	Turrialba, Costa Rica	54.6	33.0	12.4
Aug. '44	Mature petioles on 10-mo. seedl.	El Palmar, V. C., Mexico	65.9	22.1	12.0
July '44	Petiole young leaf, clone GV 21	" " "	67.3	21.3	11.4
Apr. '44	Stems, 15-month seedlings	" " "	68.6	17.4	14.0
July '44	Mature petioles, 3-yr. seedling	" " "	69.7	19.0	11.3
"	Petiole young lf., clone GA 49	" " "	70.5	18.7	10.8
Mar. '45	Two years, group B	" " "	70.7	18.0	11.3
"	" " " A	" " "	71.5	13.3	15.2
May '44	Estimated 2 years	Gomez Farias, Tps., Mex.	72.0	10.0	18.0
"	" 1½ "	" " "	73.3	10.6	16.1
July '44	Clone GA 49, stem 1 inch dia.	El Palmar, V. C., Mexico	73.7	16.7	9.6
Mar. '45	Two years, group A	" " "	75.2	13.3	11.5
"	" " " B	" " "	75.6	20.3	4.1
July '44	Clone GV 21, stem 1 inch dia.	" " "	76.9	12.9	10.2
Apr. '44	In production, estimated 20 yr.	" " "	77.7	4.7	17.6
July '44	Plantation A, 3 years	" " "	77.8	9.8	12.4
"	Seedling 1½ years	" " "	78.3	9.8	11.9
"	Plantation B, 3 years	" " "	78.4	10.8	10.8
"	In production, seedling origin	Cairo, Costa Rica	81.5	1.4	17.1
"	" "	" "	82.0	1.7	16.3
"	" "	" "	82.2	2.2	15.6
"	" "	" "	82.8	4.0	13.2
"	" "	El Palmar, V. C., Mexico	83.0	3.3	13.7
"	" "	" " " "	83.2	5.4	11.4
"	" "	" " " "	84.2	2.8	13.0
"	" "	Cairo, Costa Rica	84.3	0.6	15.1
"	" "	" "	84.9	1.6	13.5
"	" "	" "	85.7	0.4	13.9
"	" "	" "	89.0	1.1	9.9

parent correlation could be indicated though actually non-existent.   According to these analytical methods, insolubles in total solids are defined as substances which are insoluble in either acetone or benzene; and rubber hydrocarbons are benzene-soluble substances which form bromides insoluble in 95% ethyl alcohol.

From these analyses, it was found that the percentage of rubber hydro-carbons was inversely correlated with the insolubles (table I and fig. 1). The correlation coefficient between percentage of rubber hydrocarbons and
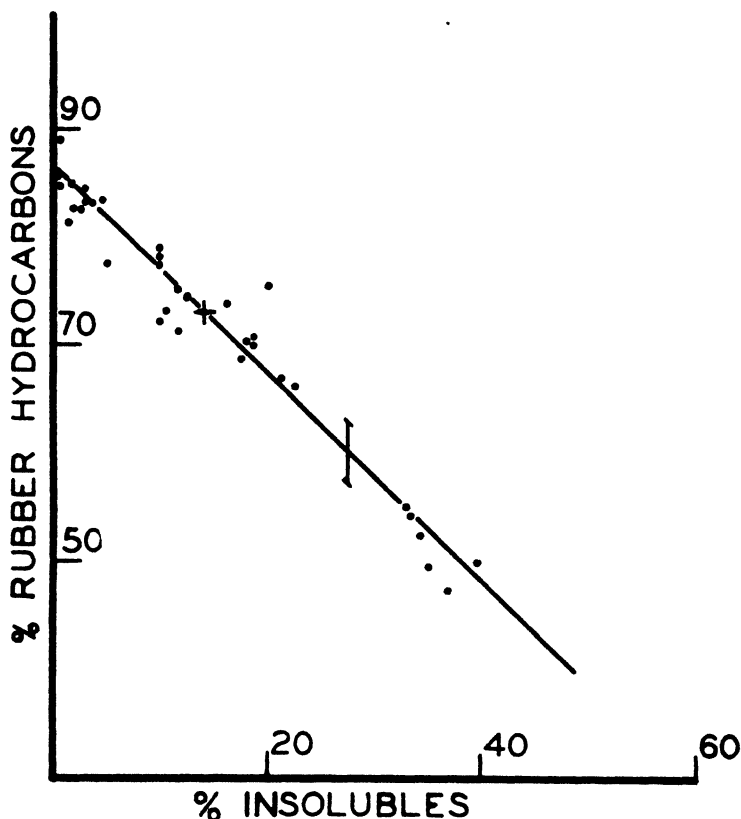
FIG. 1.   Inverse correlation between percentage of rubber hydrocarbons and insolu-bles in total solids of latex from *Hevea brasiliensis*.   Regression line with standard error calculated from 34 analyses.   Cross shows average point.

insolubles was − 0.970.   Since these analyses appear to represent a straight line relationship, it was possible, by using the method of least squares for the computation of regression averages, to calculate the equation:

$$R\% = 86.6 - 0.972\ I\%$$

where, R = rubber hydrocarbons and I = insolubles.   The line defined by this equation (fig. 1) has intercepts of 86.6 and 89.2 on the rubber hydrocarbons and insolubles axes, respectively.   The standard error of the estimate was ± 2.8%.   The regression coefficient, − 0.972, is significant at the 1% level. The corresponding regression coefficient for a similar relationship in latex from *C. grandiflora* was − 0.809.

The significance of the inverse correlation between rubber hydrocarbons and insolubles in latex from *H. brasiliensis* is not yet understood. Isolation and identification of the substance, or substances, in the insolubles which cause the correlation may indicate if this relationship is caused by the presence of rubber hydrocarbon "precursor" in the insolubles or by some other means. It is clear that the correlation affords a basis for further physiological studies on latex rubber formation. The fact that a similar relationship exists in the latex from rubber-producing plants of two different botanical families may indicate that it is of a fundamental nature.

The authors thank DR. F. J. LE BEAU and DR. T. J. GRANT for their help in making the latex collections in Mexico and Costa Rica.

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THE FORMATION OF CHLOROPHYLL A IN ETIOLATED OAT SEEDLINGS

RICHARD H. GOODWIN AND OLGA V. H. OWENS

(WITH ONE FIGURE)

The ratio of chlorophyll *a* to chlorophyll *b* has been determined for the green tissues of many species of plants. Chlorophyll *a* usually lies between 67% and 78% for the normal green tissues of higher land plants (**2, 10, 11**).

In etiolated tissues, however, chlorophyll *a* has been reported to be formed much more rapidly than *b*, when the plants are first exposed to light, regardless of the spectral region employed (**8**). Although not specifically commenting on this interesting phenomenon, FRANK (**3**) has recently published spectrophotometric data indicating that the same thing holds true for completely dark-grown seedlings of *Avena byzantium* var. *sativa*, after five-hour exposures to light. It is possible that this early rapid production of chlorophyll *a* may be correlated with the initial presence in dark-grown plants of a relatively higher percentage of "protochlorophyll *a*" than of "protochlorophyll *b*," a condition which reportedly exists in pumpkin seeds (**9**).

In studies on the production of chlorophyll in *Avena sativa* (**5**), the authors have observed an almost complete absence of chlorophyll *b* in chlorophyll extracts obtained from plants irradiated for two or three hours with visible light. Husked seeds were laid out on wet absorbent cotton and germinated in complete darkness under controlled conditions of temperature and humidity. After two and a half days, some of the seedlings were irradiated for two hours with monochromatic blue light (4358 Å) isolated with a Corning polished glass filter #5113, 2 mm. thick, a 5-cm. layer of 1.2% $CuSO_4$, and a 5-cm. layer of 0.4% quinine hydrochloride in $N/5$ HCl. The transmissions of the liquid filters have been reported elsewhere (**1**). Radiation intensity at the level of the plants was approximately 5.5 ergs per mm.^{2} per second. The shoots of the irradiated seedlings and of dark-grown controls were then removed just below the coleoptilar node and ground in pure acetone. The acetone extract was centrifuged and the pigments transferred to ethyl ether in a separatory funnel. The acetone was removed by successive washings with distilled water to which a trace of calcium carbonate had been added. Extractions were carried out in very dim light and the extracts stored in the dark for two days over solid carbon dioxide, until spectroscopic studies could be made.

Absorption spectra were obtained on an automatic recording spectrophotometer (**6**),[1] with the monochromator slit width set at 100 Å. Figure 1 shows the relative absorption per shoot of extracts from completely dark-

grown plants (curve A) and from irradiated plants (curve B) in the spectral region not obscured by carotenoid absorption. The differences in these curves are due chiefly to the synthesis of chlorophyll during the two-hour exposure. The two absorption maxima for the dark-grown extract probably
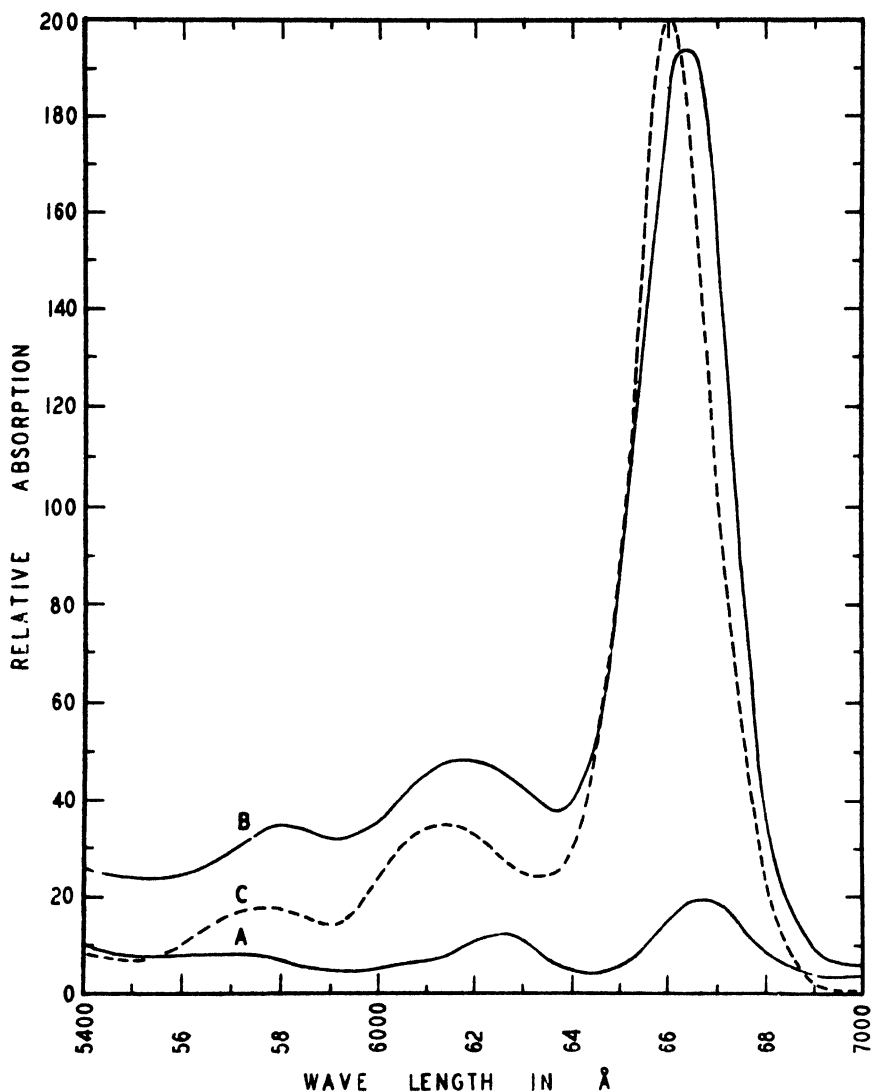

FIG. 1. Absorption spectra of ether extracts of Avena shoots and of pure chlorophyll a in ether. Curve A: extract from 429 dark-grown plants; Curve B: extract from 458 plants exposed for two hours to monochromatic blue (4358 A) light at a radiation intensity of approximately 5.5 ergs/mm.²/sec. The plants in the two groups were of comparable size; the data are expressed as relative absorption per plant. Curve C (broken line): pure chlorophyll a (4).

represent the absorption of "protochlorophyll" (6250 Å) (7) and of chlorophyll a (6670 Å). The positions of three of the absorption maxima for the extract from irradiated plants at 6640 Å, 6180 Å, and 5800 Å are in close agreement with those for pure chlorophyll a, although there appears to be a shift toward the red of about 40 Å. The absorption spectrum of pure

chlorophyll *a,* isolated from spinach by the senior author (4) following the method of ZSCHEILE and COMAR (12), was obtained on the same spectrophotometer and is shown for comparison in figure 1 (broken curve C). The maxima for this preparation at 6600 Å, 6140 Å, and 5760 Å coincide with those previously reported (12).

There is no shoulder in the absorption curve for the extract from irradiated plants at 6425 Å, the region of the chlorophyll *b* absorption maximum (12), which would be expected, if appreciable quantities of this component were present. To check whether traces of chlorophyll *b* might be present, another acetone extract from 270 plants irradiated for three hours was centrifuged, and the pigments transferred to petroleum ether (b.p. 35° to 45° C.). The petroleum ether solution was washed repeatedly with distilled water to which a trace of calcium carbonate had been added, dried over anhydrous sodium sulphate, and filtered onto a small adsorption column of powdered sucrose 2 cm. in diameter and 15 cm. high. The chromatogram, which was developed for three-quarters of an hour with petroleum ether, showed but a single blue zone above the carotenoids, no green chlorophyll *b* zone being in evidence. The chlorophyll band was mechanically removed and the pigment eluted with 10 ml. of ethyl ether. Two milliliters of the ether extract were diluted with 18 ml. of acetone, and the fluorescence of the solution, when irradiated with blue (4358 Å) and violet (4047 Å) light was measured on a Klett fluorimeter-colorimeter.[2] The ratio of the fluorescence in the violet to that in the blue was 0.175. This value is nearly that obtained for pure chlorophyll *a* (0.182 ± 0.012), and not that obtained for chlorophyll *b* (0.051 ± 0.001). Details of the fluorescent behavior of chlorophylls *a* and *b* and mixtures of these two components will be given elsewhere (4).

It is concluded that virtually no chlorophyll *b* is formed in oat plants during the first three hours of exposure to blue light.

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NOTES

The Boston Meeting.—The twenty-first annual meeting of the American Society of Plant Physiologists was held in Boston, December 27–30, 1946. Five joint sessions of the American Society of Plant Physiologists and the Physiological Section of the Botanical Society of America were held. Sixty-three papers were presented in addition to the three symposia. One symposium was given at each of the following meetings: Section G of the A.A.A.S. on Friday, December 27, Section O and the A.S.H.S. on Saturday, December 28, and Section O on Monday, December 30. The meetings were all well attended and the papers were of more than usual interest. The Saturday symposium on plant respiration was especially well received by a large audience. At the annual meeting of the Council of the American Association for the advancement of Science held in Boston, Dr. Walter F. Loehwing was elected Vice President of the A.A.A.S. and Chairman of Section G for Botanical Sciences for 1947.

The Plant Physiologists' Dinner with Dr. B. M. Duggar presiding was held at the Vendome Hotel on Friday evening, December 27 and was attended by 152 persons. The retiring president's address by Dr. Paul J. Kramer was entitled, "A Viewpoint for Plant Physiologists," in which he stressed the importance of cooperation between research workers in fundamental and applied physiology. Recipients of the Charles Reid Barnes Award and the Stephen Hales Award were announced.

An Executive Committee meeting preceded the business session of the Society held on Monday afternoon, December 30 in order to prepare the agenda for presentation. Several constitutional changes relative to corresponding members and the Program Committee were approved for presentation to the membership in March by mail ballot. These concern the privilege of corresponding members to receive the journal, as well as the bulletins, without charge. It was also recommended that the Secretary act as Chairman of the Program Committee. Questions as to the time and place preferred for the next meeting of the Society, as well as to membership reaction to present and proposed National Science Foundation bills will be presented in the mail ballot.

The Stephen Hales Award.—Dr. Burton Edward Livingston, Professor Emeritus of plant physiology at Johns Hopkins University, was awarded the Stephen Hales Award. Dr. Livingston was born February 9, 1875 in Grand Rapids, Michigan. He received the B.S. degree from the University of Michigan in 1898 and the Ph.D. from the University of Chicago in 1901. Since his graduation Dr. Livingston has been active in the field of plant science, not only as an instructor and research worker, but also as a leader in many of the professional organizations of the country. He was elected to the first Barnes honorary life membership of the American Society of

Plant Physiologists, and has served in the Society as its President, member of the Executive Committee, and member of the Editorial Board.

Release from teaching responsibilities upon retirement from his professorship in 1940 has permitted Dr. Livingston to turn his undiminished energy even more effectively to the improvement of professional organizations of importance to all scientists. His many years of executive work in the American Association for the Advancement of Science and numerous other professional organizations constitute a remarkable record of public-spirited service.

Dr. Livingston has been a pioneer investigator into the primary water relations of plants and inventor of many of the procedures which are standard in that field. Over the past half century he, through his students and through his own personal efforts, has had a remarkably great influence on the progressive development of plant physiology in this country.

The American Society of Plant Physiologists is proud to link the name of Stephen Hales, Father of Plant Physiology, with that of one who has made an equally great contribution. It was with a deep sense of fitness that the Society conferred upon Dr. Burton Edward Livingston the Stephen Hales Award.

The Charles Reid Barnes Award.—Dr. Edwin Cyrus Miller, Professor Emeritus of plant physiology at Kansas State College, was awarded the Charles Reid Barnes Life Membership. Dr. Miller was born December 16, 1878 near Baltimore, Ohio. He received the B.S. degree in 1904 and the A.B. degree in 1906 from the old National Normal University at Lebanon, Ohio. From Yale University he received an A.B. in 1907 and the Ph.D. in 1910. Since that time Dr. Miller has been a member of the staff of Kansas State College and of the Kansas Agricultural Experiment Station. Prior to his retirement last year, Dr. Miller served two years as acting head of the Department of Botany and Plant Pathology.

For forty years Dr. Miller has worked untiringly upon problems of plant physiology, especially those associated with cereal crops. His work has been remarkable for the thoroughness and care with which it has been brought to completion. Plant physiologists are indebted to Dr. Miller for many important contributions to the knowledge of water relations and carbohydrate metabolism, particularly as they apply to the corn plant.

Dr. Miller's book, "Plant Physiology," which appeared first in 1931 with the revised edition in 1938, has provided an outstanding text with extensive bibliographies for advanced classes, graduate students, and research workers in all parts of the world.

Dr. Miller has served as President of the American Society of Plant Physiologists and as a member of the Editorial Board of the journal, PLANT PHYSIOLOGY. He is a member of Sigma Xi, Phi Beta Kappa, Phi Kappa Phi, A.A.A.S. (Fellow), Alpha Zeta, Gamma Sigma Delta, and Farm House Fraternity.

In recognition of Dr. Miller's outstanding accomplishments, the high standards of his professional achievements, and his tenacity in search of answers to technical problems, the American Society of Plant Physiologists presented to him the Charles Reid Barnes Life Membership Award for 1946. With this award go the Society's sincere wishes for many happy years of active and creative work.

Mechanisms of Reactions at Carbon-Carbon Double Bonds.—CHARLES C. PRICE. Interscience Publishers, Inc., 215 Fourth Avenue, New York 3, New York. 120 pages. $2.50.

Manuring of Cotton in India.—V. G. PANSE. Indian Central Cotton Committee, Bombay, India. 63 pages. $1.50.

This book is a report of the results of cotton manurial trials carried out in India over a period of years with suggestions for future experiments. Panse, Statistician, Cotton Genetics Research Scheme, Indore, has presented the material in a logical way, including figures and tabulated data.

Books and World Recovery.—The desperate and continued need for American publications to serve as tools of physical and intellectual reconstruction abroad has been made vividly apparent by appeals from scholars in many lands. The American Book Center for War Devasted Libraries has been urged to continue meeting this need at least through 1947. The Book Center is therefore making a renewed appeal for American books and periodicals—for *technical and scholarly books and periodicals in all fields* and particularly for *publications of the past ten years.* We shall especially welcome complete or incomplete files of PLANT PHYSIOLOGY.

The generous support which has been given to the Book Center has made it possible to ship more than 700,000 volumes abroad in the past year. It is hoped to double this amount before the Book Center closes. The books and periodicals which your personal or institutional library can spare are urgently needed and will help in the reconstruction which must preface world understanding and peace.

Ship your contributions to the American Book Center, c/o The Library of Congress, Washington 25, D. C., freight prepaid, or write to the Center for further information.

PLATE I

FREDERICK FROST BLACKMAN
JULY 25, 1866–JANUARY 30, 1947

IN MEMORIAM

FREDERICK FROST BLACKMAN

JULY 25, 1866–JANUARY 30, 1947

FREDERICK FROST BLACKMAN, M.A., D.Sc. (London), F.R.S., Fellow of St. John's College, Cambridge, and formerly Reader in Botany in the University of Cambridge, was born on July 25, 1866, and died at his home in Cambridge, England, on January 30, 1947, in his 81st year.

The name of F. F. BLACKMAN has long been familiar to every student of botany as one of the most distinguished plant physiologists of his time and he is assured of a permanent place in the history of that branch of botanical science to which he devoted his life and to which he, and the school of thought he founded and led, have contributed so much during the past half century or so. Although F. F. BLACKMAN spent the whole of his scientific career at Cambridge (entering as an undergraduate in 1887, appointed Demonstrator in Botany in 1891, and later serving as Lecturer and Reader from 1904 to 1936) his influence so permeates the development of plant physiology in his time that his story belongs in a measure to plant physiologists everywhere. He received the Fellowship of the Royal Society of London in 1906, was President of Section K (Botany) of the British Association in 1908, received other honors and distinctions including a Royal Medal of the Royal Society in 1926, and was President of the Section of Plant Physiology at the International Botanical Congress of 1930. Recognizing this, the American Society of Plant Physiologists elected him their first foreign Corresponding Member in 1932, and in 1934 awarded to him the Charles Reed Barnes Life Membership. Thus while Plant Physiologists everywhere take note of the passing of a great pioneer of their science, the AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS is proud that he may be counted among its most distinguished members.

To appraise the work and influence of F. F. BLACKMAN one must look beyond the papers which bear his name, important as these were and are. His great contribution must be seen in perspective against the unfolding history of botany in his time. The strangely intermittent milestones in the early history of plant physiology in Britain are associated with particular names, familiar to every student, as those of NEHEMIAH GREW, STEPHEN HALES, JOSEPH PRIESTLEY, T. A. KNIGHT, and FRANCIS DARWIN. F. F. BLACKMAN, however, did something more over and above the discoveries identified with his name for he created the first school of plant physiology in England in the modern tradition. The significance of the life and work of BLACKMAN lies in the fact that he stimulated in England a new outlook on plant science as he led the way in applying quantitative experimental methods to the investigation of complex physiologcal processes and in for-

mulating interpretations through the application of physico-chemical reasoning.

In a measure BLACKMAN was the product of his time; his greatness lies in the fact that he responded to its challenge. The impetus of the great renaissance of biology as a practical laboratory science under Huxley at South Kensington in the 70's had been carried over into botany by THISEL-TON-DYER and by S. H. VINES. It is perhaps hard for contemporary teachers to appreciate that even in a country with such an ancient academic tradition as England, practical tuition in botany in the modern sense was unknown prior to the 70's, and plant physiology as a practical laboratory science literally did not exist. Drawing much of his stimulus from the great German physiologist SACHS, VINES was the first advocate of the new practical and experimental outlook on botany at Cambridge and in 1883 he was elected to the newly created post of Reader in Botany. VINES also compiled the first distinctively English textbook on the Physiology of Plants and seemed destined to found at Cambridge the School of Plant Physiology which did not come fully into being till later, for VINES left Cambridge in 1888 to become Professor of Botany at Oxford. The Readership as well as the fostering of the first practical teaching of Plant Physiology in the Botany School at Cambridge then devolved upon FRANCIS DARWIN. In the year 1881, as DR. F. F. BLACKMAN is quoted by F. O. BOWER as saying, "Plant Physiology may be said to have crystallized out in its pure form from the general solution of mixed botanical knowledge," for in ths year the first edition of PFEFFER's textbook appeared. This was the plant physiological scene which awaited F. F. BLACKMAN. He was appointed Demonstrator under FRANCIS DARWIN in 1891 and later succeeded him to the Readership, a post he held until 1936 when he was in turn succeeded by one of his own colleagues and students, DR. G. E. BRIGGS.

Thus the life and active career of F. F. BLACKMAN spans almost the entire modern period of plant physiology in England. Due to his example and the prestige enjoyed by the school which grew up around him, Plant Physiology in England emerged as a branch of botanical thought and discipline worthy to take its place beside the more ancient and firmly established scientific traditions. We shall probably not see his like again for the historical setting which presented him with his opportunity and its challenge will not again return. For the high standards of scholarship which BLACKMAN maintained during this formative period, and the deliberate, calm judgment which pervaded his written works, the science owes him a deep debt of gratitude. Increasingly as the years passed, and until 1936, graduates and graduate students came to be influenced by his teachings, many of them destined to attain eminence and to occupy Botanical Chairs and official positions in England, the Dominions, and in the Empire. Thus it may be said that, from the turn of the century, F. F. BLACKMAN, as no other man, influenced the trend of the subject in the English speaking world.

BLACKMAN leaves no great textbook on the pattern of PFEFFER or SACHS. The actual physiological papers which bear his name are relatively few for so long and so important a career. Plant physiologists are likely to forget that in his earlier years BLACKMAN wrote with authority on the classification and phylogeny of the algae and this interest in algae overlapped the absorbing interest of his career in a discussion of "chromatic adaptation." However, two main series of papers reveal his physiological work and his influence even when he was not avowedly an author. The first of these series began with his own pioneer work and under the series title "Experimental Researches on Vegetable Assimilation and Respiration" appeared in twenty-one successive parts from 1895 to 1933. The other, entitled "Analytical Studies in Plant Respiration," appeared in seven successive parts from 1928 to 1937. These two series go far to tell the story of the Cambridge School of Plant Physiology under F. F. BLACKMAN and, insofar as he leaves a written legacy, we find it here. However, BLACKMAN was always deliberate in his writing and it is common knowledge in Cambridge and in England that much original work done at Cambridge during his period of tenure never reached the stage of publication. This loss to the larger community of plant physiologists can only be offset by the calm, matured judgment that pervades all the papers prepared under the influence of BLACKMAN's mind.

Reading his papers one is struck with the clarity of his formulations and his ability, where necessary, to rise above the tedious detail out of which great conclusions must often arise. Withal he maintained a dignity of style and language often lacking in our modern reporting of experimental findings and theoretical conclusions. In rereading these early works one comes unexpectedly across occasional touches which seem to carry down the years a breath of the Victorian and Edwardian England in which BLACKMAN moved and worked. His first published papers, communicated to the Royal Society of London by FRANCIS DARWIN in 1884, were really extended notes anticipating by a year in the Proceedings fuller presentations in the Philosophical Transactions. These notes and papers described "A New Method for Investigating the Carbonic Acid Exchange of Plants" and its application to an investigation "On the Path of Gaseous Exchanges between Aerial Leaves and the Atmosphere."

In this first work the method was one which was to be repeated several times as the long series unfolded. First a careful elaboration of a new technical procedure, with painstaking attention to detail, the technique was thoroughly evaluated and carefully described followed by the presentation, in an accompanying paper, of the mature investigation as it was finally carried out. For its time BLACKMAN's first apparatus was unusually elaborate, but he disarmingly states that: "Simplification of technique by complication of apparatus has been the guiding principle and the result is that, although the whole consists of at least eight separate pieces of apparatus, many of them being further in duplicate and all connected together by a

plexus of tubes, yet the working is so automatically arranged that the operator, beyond reading the burettes and occasionally working a finger bellows, has nothing to do but turn stopcocks.''

BLACKMAN showed how by his new technique he could determine the carbon dioxde absorbed or released by a small area of a leaf surface or by a single seed and how he used the method to test whether gas exchange occurred through stomata or through the cuticle. Quantitative data were critically marshalled to show that both CO_2 evolved in the dark and assimilated in the light passed predominantly through the stomata. The cuticular theory had been advocated by BOUSSINGAULT who found that ''leaves of *Nerium oleander* assimilated less when the upper astomatiferous surface had been coated with an unguent than when the lower stomatiferous surface had been so coated.'' BLACKMAN showed conclusively that BOUSSINGAULT's conclusion flowed from his use of such high concentrations of CO_2 (30%) that when this penetrated freely through the open stomata it inhibited assimilation whereas in the leaf with stomata almost completely blocked, the internal concentraton still allowed assimilation to proceed.

The two subsequent papers in the series published in 1904–1905 recorded work of MISS MATTHAEI on the effect of temperature and illumination on the assimilation of carbon dioxide by leaves of cherry laurel (*Prunus laurocerasus*) and Jerusalem artichoke (*Helianthus tuberosus*). Here the emphasis was upon the interacton of the various factors which affect photosynthesis and upon the fact that under conditions of natural illumination it is the carbon dioxide concentration of the atmosphere which controls the rate of assimilation. Doubtless this phase of the work prompted BLACKMAN's most famous dictum, enunciated as the Principle of Limiting Factors in 1905 in the Annals of Botany (p. 289), in the following stately passage: ''When a process is conditioned as to its rapidity by a number of separate factors the rate of the process is limited by the pace of the slowest factor''; and again, ''When the magnitude of a function is limited by one of a set of possible factors, increase of that factor, and of that one alone, will be found to bring about an increase of the magnitude of the function.'' It matters little whether or not later investigators believe that the principle holds as rigidly as originally conceived. The importance of the principle so simply enunciated lies in the guidance it gave to the analysis and interpretation of experimental data obtained on systems upon which two or more variables interact.

It would be tedious to recapitulate the familiar trend of these researches. The series included papers by THODAY; with A. M. SMITH on photosynthesis in submerged aquatics; by BRIGGS, on the development of photosynthesis in seedlings (a subject studied earlier in BLACKMAN's laboratory with reference to etiolated seedlings by MISS IRVING); by WILMOTT, who elaborated and improved upon the familiar bubble counting technique with Elodea; by MASKELL, who investigated diurnal rhythm in the assimilation of leaves and made a critical study of assimilation in relation to stomatal opening;

by JAMES, who reinvestigated the dependence of photosynthesis upon carbon dioxide supply using submerged waterplants; and again by BRIGGS, upon the energetic efficiency of photosynthesis in green plants. BLACKMAN'S recognition of slowly progressing thermal or "dark" reactions in photosynthesis which at high light intensity or high carbon dioxide concentrations cause the rate of the overall process to respond to temperature as do chemical reactions is acknowledged by the now general use of the term "Blackman Reaction" to describe this aspect of carbon assimilation.

Analytical studies in plant respiration opened with three papers; two in collaboration or association wth PARIJA and one by F. F. BLACKMAN himself. The new series devoted a great deal of attention to the respiratory behavior of whole, stored apples and, later, of potatoes. The staple technique was again the determination of respired carbon dioxide. The time drifts in the respiratory behavior of stored apples were revealed as well as the changes which ensue when apples are transferred from air to oxygen or nitrogen. On this and similar evidence BLACKMAN formulated a general scheme of plant respiration and enunciated his concept of "Oxidative Anabolism." This was a device to explain the relation between the carbon dioxide evolved in air and in nitrogen. Accepting that the path of carbohydrate breakdown is probably common to both aerobic and anaerobic respiration (at least as far as three carbon intermediates), BLACKMAN postulated that in air part of these intermediates are metabolized to carbon dioxide and water and part resynthesized to products which become reavailable for respiration. In nitrogen, on the contrary, the whole of the three carbon intermediates are utilized to form the products of fermentation. In this concept BLACKMAN followed, or was influenced by, the similar views expressed by MEYERHOF for muscle and yeast, though it must be confessed that the supporting body of biochemical evidence for the systems with which he was concerned was not strong. BLACKMAN's concept of "Oxidative Anabolism," like the MEYERHOF view, has not passed unchallenged and in particular its generality has been called into question. However, this brings BLACKMAN's contributions close to the more controversial fields of the present day. The practical science he did so much to foster has moved far from the days when exact measurements of overall gas exchange had yet to be made in order that the problems and processes of plant respiration and photosynthesis could be visualized. Other hands and minds must now thread the way through the often bewildering maze[1] of enzymes and substrates, coenzymes and cofactors, phosphorylations and dephosphorylations, and the sequence of interlocking cyclical processes which seem necessary to explain the biochemistry of these vital processes. F. F. BLACKMAN cleared the way for this task; it will be the simpler because he built so surely.

But what manner of man was F. F. BLACKMAN? Only those who enjoyed close contact with him are entitled to speak here though it is evi-

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[1] What Blackman referred to in 1905 as "a congeries of enzymes, a colloidal honeycomb of katalytic agents. . . ."

dent that only a mind of great strength and a rare spirit could have exerted so profound an influence over the long succession of students, collaborators, and colleagues. Perhaps we may be permitted to quote from one who knew him and who writes: "He lived a full and balanced life; his devotion to science did not prevent him from cultivating other interests which included music, architecture, and painting. To problems in all spheres BLACKMAN brought a calm and dispassionate mind and a habit of careful and patient discrimination. There was an unobtrusive strength in his personality which seemed to derive from the blending of wisdom, subtlety of perception and gentleness.

"BLACKMAN was a great teacher as well as a gifted investigator and he linked closely together the two functions of teacher and research. His advanced lectures were based largely upon experimental results obtained in his own school and many of these were (and still remain) unpublished. Each lecture was a perfect presentation in which BLACKMAN succeeded in imparting to his hearers a point of view as well as an ordered array of facts illustrating a particular theme. To attend his lectures was a privilege never to be forgotten and an experience which formed an important part of the discipline of the school of research which grew up under his inspiration."

The AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS therefore joins with botanists and plant physiologists everywhere and pays its tribute to the memory of DR. F. F. BLACKMAN, a great pioneer in the modern experimental approach to the study of plants and plant behavior.

The Editor is indebted to DR. F. C. STEWARD and the Memorial Committee of the Society for the preparation of this memorial biography.

STUDIES IN THE METABOLISM OF CRASSULACEAN
PLANTS; THE EFFECT UPON THE COMPOSITION
OF *BRYOPHYLLUM CALYCINUM* OF THE FORM
IN WHICH NITROGEN IS SUPPLIED

George W. Pucher, Charles S. Leavenworth, Wanda
D. Ginter, and Hubert Bradford Vickery

(WITH EIGHTEEN FIGURES)

In general, the experimental procedure used to determine the availability of nitrate or ammonium ions to plants has involved culture solutions that provide but one of the two ions. In experiments of this sort in recent years, care has been taken to maintain the pH of the solution within a range found appropriate for absorption by plants. The work in this field by Dr. J. W. Shive and his associates at the New Jersey Agricultural Experiment Station has been outstanding; Tiedjens and Robbins (11), for example, have shown that tomato and soybean plants grown at pH 7.9 in ammonium salt culture solution, or at pH 4.0 in nitrate solution, were almost equally well developed.

The effect upon the detailed chemical composition of tomato plants grown under the two conditions has been studied in this laboratory by Clark (2), who observed that the organic acid content of leaves and stalks was greatly depressed in plants grown in ammonium salt solution as compared with those grown in nitrate solution. Protein and several soluble nitrogenous components were, on the other hand, increased in the plants grown on ammonium salts, the production of glutamine in particular being enormously stimulated. Detailed comparison of the effects of the two different nutrient conditions was made difficult, however, by the fact that the plants grown with ammonium salts were much smaller than those grown with nitrate, although both were setting fruit at the time of harvest.

In order to follow more closely the effect upon tissue composition of nitrate *versus* ammonium salt nutrition, it is desirable to grow the plants in a series of culture solutions of similar over-all composition, but with the relative proportion of nitrate to ammonium ions varied in regular steps from all-nitrate nitrogen to all-ammonium nitrogen. Such an experiment

was carried out a few years ago in this laboratory upon the tobacco plant (16). Plants grown on a culture solution 20% of the nitrogen of which was present as ammonium ion (the balance as nitrate ion) were stimulated with respect to those that received all of their nitrogen as nitrate ion, being larger and better developed. Plants that received 40% and 60% of their nitrogen as ammonium ions were approximately of the same size as the all-nitrate plants, as measured by the organic solid content, and could therefore be closely compared with respect to chemical composition. Plants that received 80% and 90% of the nitrogen as ammonium ions were successively smaller; nevertheless, the data for composition in terms of grams per plant fell upon smooth curves when plotted against the composition of the culture solution, and comparisons upon a concentration basis of any of the components were apparently valid throughout the series.

As in the case of Clark's experiments with the tomato plant, the outstanding differences, as the relative proportion of the nitrogen supplied as ammonium ions increased, were in the composition with respect to organic acids and in certain of the soluble nitrogenous components. Organic acids decreased in amount per plant and in concentration, especially in the case of malic acid, to a fraction of that present in the nitrate plants; distribution of the organic acids also varied greatly. In short, plants grown with a high proportion of the nitrogen supplied as ammonium salt were, in many details of their chemical composition, entirely different from those grown with a high proportion of nitrate. These results suggest the importance, in connection with the present investigation of crassulacean plants, of studying the effect upon the organic acid composition of the step by step replacement of nitrate in the culture solution by ammonium salts. Plants of this family differ from the more thoroughly investigated species such as tomato, tobacco, and rhubarb, not only in over-all organic acid composition but also in diurnal variation in organic acid content. They therefore possess an unusually active organic acid metabolism.

This report contains results of the analysis of leaves and stems of *Bryophyllum calycinum* plants grown with the aid of a series of culture solutions, the first of which provided all nitrogen as nitrate ion, the second and third, 50% and 75%, respectively, of the nitrogen as ammonium ion (the balance being nitrate ion), and the last of which provided all nitrogen as ammonium ion. The other components of the solutions were held constant, the sulphate ion alone being increased in proportion to the increase in ammonium ion. In addition, the effect of a potassium-deficient culture solution was studied in order to see what, if any, influence the lack of an adequate supply of potassium would exert upon the composition of the tissues. This solution contained about 14% of its nitrogen as ammonium ion, the balance as nitrate.

Growth of the plants

Culture solutions were prepared with the composition shown in table I. Stock solutions of each of the salts were provided in 0.5 M concentration

TABLE I

COMPOSITION OF CULTURE SOLUTIONS.   (FIGURES ARE MOLARITY OF THE SOLUTION AS USED)

SALTS	PERCENTAGE OF NITROGEN AS AMMONIUM NITROGEN				POTASSIUM-DEFICIENT
	0%	50%	75%	100%	
KH_2PO_4	0.001	0.0031	0.0031	0.0031	
$Ca(NO_3)_2$	0.00225				0.0021
$MgSO_4$	0.0014	0.0014	0.0014	0.0014	0.0014
KNO_3	0.0021				
$Mg(NO_3)_2$	0.0043				0.0043
NH_4NO_3		0.0076	0.0038		
$CaSO_4$		0.00225	0.00225	0.00225	
$(NH_4)_2SO_4$			0.0038	0.0076	
$NH_4H_2PO_4$					0.0021

and the culture solutions were made up in 10 liter lots with tap water. Boron and manganese salts, sufficient to provide 1 p.p.m. of these elements in the final volume, and enough of Hoagland's A–Z solution to provide 0.01 to 0.02 p.p.m. of the minor elements were added before dilution. The reaction was finally adjusted to pH 5.8 with sulphuric acid. Each culture solution contained 212.8 mg. of nitrogen per liter.

The plants were grown as described in the first paper of this series (8); on March 20, 1941, 25 young plants were transplanted into washed sand in one-gallon crocks and allowed to establish themselves for nine days, a nitrate-containing culture solution being provided. At this time, five sets of two plants each, selected for uniformity of size, were flushed with water until nitrate could no longer be detected in the effluent; they were then treated in a series with the culture solutions, the drip method of Shive being employed. After 46 days, it became more convenient to add the culture solution in portions of 500 ml. every other day.

There was no marked difference in the behavior of the plants until after 42 days when the 100% ammonia plants began to develop yellow mottling of the leaves and brown tingeing of some leaf margins. At intervals during the course of the experiment, samples of sand were taken from the crocks by means of a cork-borer and the pH of an aqueous suspension was determined. The results suggest that the changes in the appearance of the leaves did not arise from undue acidity of the cultures (table II). The plants were har-

TABLE II

OBSERVATIONS OF PH OF SUSPENSIONS OF SAND IN WHICH PLANTS WERE GROWING

NUMBER OF DAYS	PERCENTAGE OF NITROGEN AS AMMONIUM NITROGEN			
	0%	50%	75%	100%
0	5.8	6.0	5.8	6.0
42	5.9	5.1	5.2	4.9
57	5.9	5.0	5.1	4.6
70	5.5	5.1	4.5	4.7

vested after 70 days on June 9 at 8:00 A.M., the time of day being chosen to provide leaf tissue still considerably enriched in organic acids.

The nitrate plants were obviously healthy, the leaves being fully green. The lower leaves of the 50% ammonia plants were slightly mottled with yellow; those of the 75% ammonia plants showed this effect extending somewhat higher up the plant and the edges of the lower leaves were reddish yellow in color. The 100% ammonia plants bore leaves all of which, except the topmost, were yellow mottled with reddish brown edges, while the plants themselves were definitely smaller than the others. The potassium-deficient plants likewise showed mottling of the lower leaves while those in the middle region were yellowish in color and withered at the edges. Only the top leaves were fully green. Many of the leaves were curled down at the tips and up and inward at the edges.

TABLE III

FUNDAMENTAL DATA ON COUNTS AND WEIGHTS OF SAMPLES OF *Bryophyllum calycinum* PLANTS GROWN FOR 70 DAYS. (FIGURES ARE THE TOTAL FOR TWO PLANTS)

PERCENTAGE OF NITROGEN AS AMMONIUM NITROGEN	LEAVES			STEMS		PETIOLES	STEMS AND PETIOLES	
	No.	FRESH WEIGHT	CRUDE DRY WEIGHT	LENGTH	DIAM-ETER	No.	FRESH WEIGHT	CRUDE DRY WEIGHT
		gm.	*gm.*	*cm.*	*cm.*		*gm.*	*gm.*
0	131	618.4	65.2	108	1.5	40	222.9	19.1
50	133	517.0	53.7	103	1.4	40	180.0	14.0
75	128	463.7	48.3	104	1.3	40	158.6	13.7
100	121	341.6	38.6	90	1.1	40	102.4	12.1
POTASSIUM-DEFICIENT	114	481.9	52.4	91	1.3	40	130.2	10.5

Dissection of the plants and preparation of the tissues for analysis were carried out as previously described. The record of counts and weights of leaves and stems (table III) shows the progressive effect upon the size of the plants of the substitution of nitrate by ammonium ion. It is to be noted that the number of leaves and the height of the plants were not greatly influenced, although the fresh and dry weights were. In most instances the two plants forming each sample differed from each other by less than 5%; however, the difference between the fresh weights of the two 50% ammonia plants was about 9%.

Expression of analytical data

The analytical data shown in the figures are expressed as grams per single plant in the lower part of most of the diagrams and, in concentration units, as grams per kilo of fresh tissue weight in the upper part. The points are plotted as open circles. The composition of the leaf tissue is shown by the solid lines, that of the stem tissue by broken lines. The scale

of abscissas is uniform throughout and shows the percentage of the nitrogen present in the culture solution as ammonium ion.

The composition of the potassium-deficient plants is plotted by points placed at 14% on the scale of abscissas since this culture solution contained 14% of its nitrogen in the form of ammonium ion. However, these points are not connected by lines to the others since this culture solution differed in composition with respect to both potassium and phosphorus, and the composition of the potassium-deficient plants cannot be regarded as a continuous function of the ratio of the forms of nitrogen in the other solutions. The composition of the leaf tissue of the potassium-deficient plants is shown by filled circles, that of the stem tissue by half-filled circles. Many cases will be noted in which these points lie at a considerable distance from the plotted lines showing the composition of the plants in the ammonia series.

Organic solids, water, and ash

Figure 1 shows the organic solids. The curve for the leaf in terms of grams per plant follows a straight line course, within the limits of error of the determinations, and the 100% ammonia plants contained approximately 63% as much organic material as the 100% nitrate plants. The curve for the stems follows a similar course, the decrease being to about 75% of the quantity in the nitrate plants. The potassium-deficient plants contained appreciably less organic solids both in leaf and stem than would correspond to their position on the scale of percentage of ammonium nitrogen supplied; the leaves contained 83% and the stems 64% as much organic solids as those of the nitrate plants.

The effect of alteration of the form of nitrogen in the culture solution upon the concentration of organic solids in terms of fresh weight is shown (upper part of fig. 1). The 50% ammonia plants were similar to the nitrate plants, but the curves for leaf and stem turn sharply upward as the proportion of ammonia nitrogen was increased beyond this point. The explanation of the increase in the concentration of organic solids coupled with a decrease in the actual quantity present is to be found in the behavior of the water (fig. 2). The change was not quite linear, but water in the leaves dropped from 278 gm. to 153 gm. per plant, that of the stems from 104 gm. to 45.6 gm. over the series. A relationship that is helpful in visualizing the nature of these changes is the hydration factor, that is: the number of grams of water associated with 1 gram of organic solids (table IV).

TABLE IV

HYDRATION FACTOR OF BRYOPHYLLUM TISSUES. (GRAMS OF WATER PER GRAM OF ORGANIC SOLIDS)

TISSUE	PERCENTAGE OF NITROGEN AS AMMONIUM NITROGEN				POTASSIUM-DEFICIENT
	0%	50%	75%	100%	
Leaves	10.3	10.3	10.1	9.0	9.6
Stems	14.8	14.3	12.5	10.7	13.6

The ratio between water and solids in the leaves remained constant until all the nitrogen was being supplied as ammonia; in the stem, however,
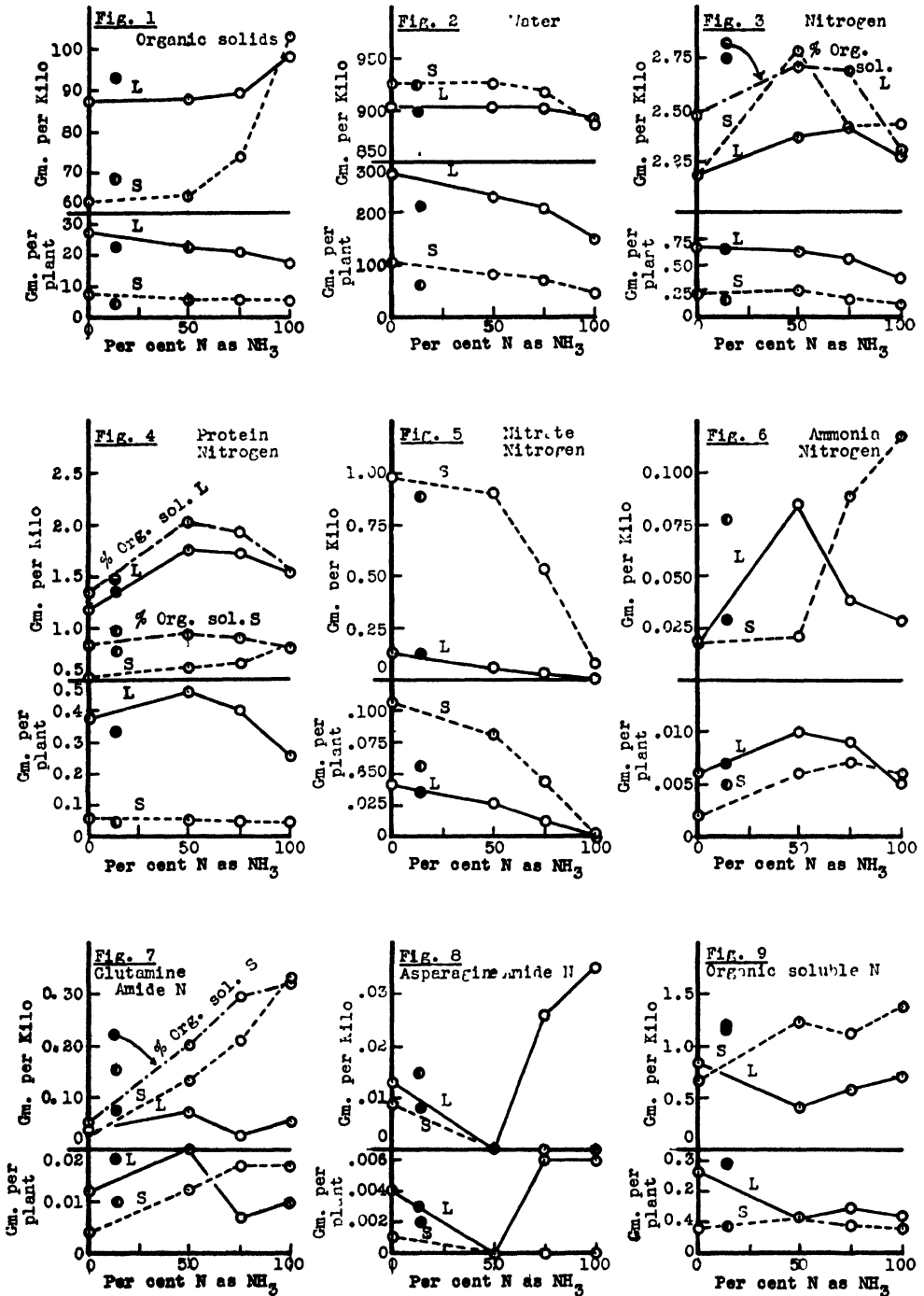
FIGS. 1–9. Analytical data for organic solids, water, and nitrogenous components.

the ratio had begun to drop at the 50% ammonia level and the total change was much greater than in the leaf. Thus, in the leaf, the change in water content (per plant) kept step with the change in quantity of organic solids;

in the stem, the character of the organic substances changed in such a way that the water-holding capacity was greatly diminished. There was also a change in the potassium-deficient plants although it was not great in the leaves. Thus, change in water-holding capacity is not specifically correlated with ammonia nutrition.

A substantial part of the change in solids of the leaf tissue can be accounted for in terms of a diminution of organic acids with increase in the proportion of ammonia in the culture solution, but only a small part of the change in the stem can be so explained. Because organic acids themselves would not be closely concerned with water-holding capacity, to this extent the failure of the leaves to exhibit gross change in hydration factor is comprehensible. The alteration in composition of the stem tissue must, however, have been directly concerned with components that are involved in the degree of hydration of the tissue. It should perhaps be pointed out that the behavior of the stem tissue cannot be accounted for in terms of the capacity of the roots of these plants to absorb water. All the plants were turgid and the leaves even of the ammonia plants were doubtless transpiring.

Concentration of the water in terms of fresh weight is shown in figure 2. The leaves remained practically constant in composition throughout the series, containing just over 90% water. The value dropped to 89.4% only in the 100% ammonia plants. On the other hand, the stem was already detectably affected when the proportion of ammonia reached 75%, the range in water content over the entire series being from 92.8% to 88.9%. It will be noted that the points for the potassium-deficient plants fall on the curves for concentration of water in the tissue but below the curves for quantity per plant.

The ash content of these plants is not plotted. The change was a linear one from 3.5 gm. per plant in the leaves of the nitrate plants to 1.26 gm. in the 100% ammonia plants. The corresponding figures for the stem are 0.95 gm. and 0.37 gm. per plant. The change in concentration was likewise linear, dropping from 11.2 gm. to 7.38 gm. per kilo in the leaves and 8.56 gm. to 7.23 gm. per kilo in the stems.

Nitrogenous components

The behavior of total nitrogen of the tissues in response to the change in form in which the nitrogen was supplied is shown in figure 3. The quantity of nitrogen in the leaves diminished as the proportion of ammonia increased in the culture solution, the drop at the highest ammonia level being quite sharp. The change was not exactly proportional to the change in the quantities of organic solids; in the upper part of figure 3, the percentage of nitrogen in the organic solids is shown (dash and dot line; ordinates to be read as percentage). This curve would be a horizontal straight line if strictly proportional changes had taken place. Actually, the curve rises and falls, showing that, at intermediate levels of ammonia nutrition, the leaf tissue solids were relatively enriched in nitrogen.

The curve for the quantity of nitrogen in the stems rises slightly at the 50% level and then falls. This effect is shown more clearly in the upper part of the figure by the curve for the concentration in terms of fresh weight. The percentage of nitrogen in the organic solids of the stem is not plotted since most of its course falls outside the scale of the diagram; the organic solids of stems of the nitrate plants contained 3.5% nitrogen while those of the 50% ammonia plants contained 4.3%. The proportion then dropped to 2.4% in the 100% ammonia plants. Thus, although the actual quantity of nitrogen in the stems of these plants did not change greatly but followed a smooth diminishing curve, the changes in relation to the fresh weight and especially to the organic solids were quite large. Much of the change in the nitrogenous composition of the stem tissue arose from the variation in nitrate nitrogen content occurring as a result of the alteration in composition of the nutrient solution; the profound drop in nitrate content had little effect upon the organic solids but greatly influenced the relative distribution of nitrogen in other forms.

Protein nitrogen is plotted in figure 4. The curve for the leaves shows a substantial increase in protein content at the 50% level of ammonia and a small one at the 75% level as compared with the nitrate plants. The smaller 100% ammonia plants contained less leaf protein than the nitrate plants. Leaves of the potassium-deficient plants contained a quantity of protein that places the point well below the curve for the ammonia series. However, the point is exactly on the curve for concentration in terms of fresh weight in the upper part of the figure. The quantity of protein nitrogen in the stem was scarcely affected by the form of nitrogen in the culture solution, the curve being nearly a straight line with a slight downward slope.

In the upper part of figure 4, data for protein nitrogen are plotted as grams per kilo and also as percentage of organic solids (dash and dot line; ordinates to be read as percentage). There was a marked enrichment of the leaves in protein at the 50% ammonia level and, at higher proportions, there was only a moderate diminution in the concentration of protein whether measured in terms of fresh weight or of organic solids.

The apparent behavior of the protein nitrogen of the stem differs according to the method of expression. In terms of grams per kilo, there was a continuous increase along a smooth curve that became steeper in slope at high proportions of ammonia. In terms of organic solids, there was an increase followed by a decrease. The difference between the two curves is probably attributable mainly to the difference in hydration factor of stem tissue resulting from the increase in proportion of ammonia in the culture solution.

The general picture of behavior of the protein nitrogen in these plants suggests that a moderate proportion of ammonia in the nitrogen supplied by the culture solution is favorable for the synthesis of protein in leaves. This conclusion is emphasized by data for the soluble organic nitrogen (fig. 9). Protein nitrogen was increased at the 50% level although the organic

soluble nitrogen decreased; this suggests that the 50% ammonia culture solution was more efficiently utilized for the synthesis of protein in the leaves than were either the 100% nitrate or the 100% ammonia solutions. An analogous case has been observed in the tobacco plant (16); here a substitution of 20% of the nitrate in the culture solution by ammonia gave rise to a larger plant with an increased quantity of protein in the leaves. In this case, also, the *concentration* of the protein in terms of organic solids was increased, but the concentration in terms of fresh weight was not. Protein nitrogen in the leaves of the potassium-deficient plants was a little low in comparison with the others; that in the stem, however, was almost in line.

The quantities of nitrate nitrogen are shown in figure 5. The stem tissue was far more enriched in this component than the leaf, the difference between the two being emphasized by data calculated on a concentration basis. Both tissues show a relatively smooth progression in the quantities of nitrate nitrogen contained, the curve for the leaf being nearly a straight line dropping almost to zero at the 100% ammonia level. Nitrate ion was, however, detectable in both leaf and stem of these plants, possibly having arisen as a result of a slight nitrifying action in the sand in which the plants were grown. Nitrate nitrogen in the leaves of the nitrate plants accounted for 6.1%, that of the stems for 44.5%, of the nitrogen contained in these tissues. These proportions dropped to 1.5% and 1.0% in the 100% ammonia plants.

Nitrate nitrogen in the potassium-deficient plants falls close to the curve of concentration for both leaf and stem of the other plants, but the actual quantity in the stem was much less than would have been expected from the relation of ammonia to nitrate in the culture solution. This was not true for the leaf; in this tissue, the point falls almost on the curve for the ammonia series of plants. The explanation may be that stems of the potassium-deficient plants were proportionately smaller both in fresh weight and organic solids. Behavior of the nitrate in this series of plants was analogous to that observed in tobacco plants (16). Although, in tobacco, the leaf rather than the stem is the organ in which nitrate mainly accumulates, a progressive diminution in the quantities of nitrate was likewise observed.

The behavior of ammonia nitrogen in the plants is shown in figure 6. The quantities present were much smaller than were those of nitrate, the scale of figure 6 being ten times larger than that of figure 5. The curves are not the converse of those for the nitrate nitrogen as might be anticipated, and it is clear that Bryophyllum, unlike tobacco, is not a plant that can be induced to store any considerable quantity of ammonia even when all of its nitrogen is supplied in this form. The curve for the quantity present in the leaf rises to a maximum at the 50% level of ammonia nutrition, that for the stem to a maximum at the 75% level and both then decrease. The curve of concentration in the leaf also rises to a maximum at the 50% level, but the 100% ammonia plants contained little more than the 100% nitrate

plants.   Only in the stem is there a progressive increase in ammonia con-
centration, but this begins at the 50% level.   The impression is given that
ammonia absorbed by the plant is promptly assimilated into other forms,
especially in the leaf.   The stem, however, is the chief locus of such storage
of excess ammonia over the needs of the plant as can be demonstrated; this
is true also of nitrate.   The potassium-deficient plants also appeared to be
able to store ammonia in the stem; the point in figure 6 which shows the com-
position of these plants is located well above the curve for the ammonia
plants.   Owing to the large scale of this diagram, however, the position of
the point is somewhat misleading; as these plants contained only about 3 mg.
of ammonia nitrogen more than the nitrate plants, the difference is less sig-
nificant than it appears.   The presence of a little excess free ammonia in the
stems of the potassium-deficient plants cannot be attributed to any serious
interference with the capacity to synthesize protein.   Concentration of pro-
tein in the stems was greater than in the ammonia series of plants (fig. 4).

The influence of ammonia nutrition upon the glutamine metabolism of
the plants is shown in figure 7.   In the leaves, the quantity of glutamine fluc-
tuated in an irregular manner which did not suggest an effect that pro-
gressed with increasing intensity as the proportion of ammonia in the cul-
ture solution was increased.   The level of glutamine in the leaves of the
nitrate plants was low, being 0.4% of the organic solids and increasing to
0.9% in the 50% ammonia plants, subsequently diminishing.   If there was
a tendency for increased synthesis at the higher ammonia levels, it was not
expressed in the composition of the leaf tissue, possibly because the glutamine
was utilized for other purposes; certainly no striking storage phenomenon
could be demonstrated.   In the stem, on the other hand, there was evidence
of progressive synthesis and storage of glutamine under the influence of
ammonia nutrition.   The quantity per plant increased along a smooth curve
up to the 75% level of ammonia in the culture solution and then remained
constant; in terms of grams per kilo of fresh weight, the curve also showed
a smooth progression, in this case extending to the 100% ammonia plants.

In order to illustrate the order of magnitude of the effect, a curve for
the quantity of glutamine amide nitrogen as percentage of organic solids
(dash and dot line; ordinate to be read as percentage) is also plotted (fig.
7).   These figures, if multiplied by the factor 10.4, give the percentage of
glutamine in the organic solids; the stem tissue of the nitrate plants con-
tained 0.6% glutamine, and the proportion increased along the curve indi-
cated to 3.3% of organic solids of the stems of the 100% ammonia plants.
In these plants, therefore, glutamine had become a highly significant com-
ponent of the stem tissue and storage was clearly evident.

This behavior is analogous to that of the stem tissue of tomato plants
(2) grown on ammonium salts and also to that of the root tissue of the beet
plant (13) after treatment with ammonium salts.   In both of these species,
observations of from 2% to 5% of glutamine have been recorded.   On the
other hand, there is a marked contrast with the behavior of the tobacco

plant which did not respond in this way. It is of interest to note that the potassium-deficient plants also were relatively enriched in glutamine, particularly in the stem. This is a result that merits further study.

The metabolism of asparagine in Bryophyllum is quite different from that of glutamine. Quantities present under any conditions of nutrition are minute (fig. 8), and none whatever was detected in either the leaves or the stems of the 50% ammonia plants, although there was a small amount in both tissues of the nitrate plants. As the proportion of ammonia in the culture solution was increased beyond 50%, a little asparagine was formed in the leaves but none appeared in the stems. This species is thus quite different from tobacco, the stem of which showed an increase in asparagine when the plants were grown under similar conditions. However, even tobacco plants grown with most of their nitrogen (*i.e.*, 80% and 90%) supplied as ammonia contained only traces of asparagine in the stem, although the leaves were somewhat enriched in this component.

These results suggest that the metabolic functions of the amides glutamine and asparagine are not the same in Bryophyllum. This conclusion seems valid also for the beet and tobacco plants. The differences in response of various species to impressed conditions that provide an increased concentration of ammonia in the cells have been interpreted (15) in terms of the relative availability of the non-nitrogenous precursors required for synthesis of the respective amides. These are assumed to be oxaloacetic acid in the case of asparagine and α-ketoglutaric acid in the case of glutamine and are supposed to arise in the course of the enzymatic transformations of organic acids summed up under the term, "respiration." Thus, the nature of the amide formed under any given set of conditions is a function not only of the supply of ammonia but also of the details of reactions concerned with respiration. Although, admittedly, this concept is vague, it is more broadly useful when an attempt is made to account for the behavior of amides in plant tissues, than is the "detoxication" hypothesis of PRIANISHNIKOV (7) which assumes that the main function of the amides is to maintain the concentration of ammonia in the tissues at a low level.

Changes in the organic soluble nitrogen are shown in figure 9. In the leaves, there was a sharp fall in soluble nitrogen at the 50% level of ammonia nutrition, and reference to figure 4 shows that this was accompanied by an increase in the amount of protein. Both changes are also conspicuous in the curves for concentration in the upper parts of the figures, and it is tempting to assume that at this point in the series a more efficient protein synthesis became possible, and to suppose that there is a cause and effect relationship. However, as the proportion of ammonia in the culture solution was further increased, there was only a small increase in the amount of soluble nitrogen and this was coupled with a marked decrease in protein. Thus the position of the equilibrium between protein and the soluble components from which protein is presumably synthesized in the cells is strongly influenced by the composition of the culture solution with respect to the form of nitrogen supplied.

In the stem, the quantity of organic soluble nitrogen remained nearly constant throughout. The concentration, however, increased over the entire series although, at the 50% point, there was a disproportionately large increase. These changes, when compared with the behavior of the protein (fig. 4), show progressions again in opposite directions, as was noted in the leaf, although the magnitudes are smaller.

The qualitative composition of the soluble nitrogen fraction in the stem must be quite different from that of the leaf. For example, the proportion of the organic soluble nitrogen present as the nitrogen of glutamine ranged from 10.7% in the nitrate plants to 47.8% in the 100% ammonia plants and, when plotted, was found to follow a curve that did not depart greatly from a straight line; in the leaf, the proportion followed an irregular course ranging from 9% in the nitrate plants to 35% at the 50% level but dropped to 16% in the 100% ammonia plants. Unfortunately, a more detailed analysis of the composition of the organic soluble nitrogen was not attempted and conclusions regarding the proportions of amino acids in the leaf and stem, and of the effect of ammonia nutrition upon these components cannot be drawn. It is clear, however, that notwithstanding the striking increase in glutamine and ammonia in the stem, the over-all increase in quantity of soluble nitrogen was still greater. There was no effect analogous to the behavior of such tissues as the hypocotyl of certain varieties of etiolated lupine seedlings in which asparagine accumulates to such an extent that it alone accounts for the greater part of the soluble nitrogen (10).

Organic acids

The total organic acids present in this series of Bryophyllum plants are plotted in terms of milliequivalents per plant and milliequivalents per kilo of fresh tissue (fig. 10). Changes in these components were major ones, and as will.be seen, followed what seems to be a general rule, namely, that increase in the proportion of ammonia in the culture solution in which the plants are grown is accompanied by a marked decrease in the amount and concentration of at least some of the organic acids present in the tissues. In the present case, the decrease followed a moderately steep straight line curve for the leaf tissue. The acids of the stem, on the other hand, although they decreased in absolute quantity and by as much as 50% of the amount present in the nitrate plants, increased slightly in concentration as calculated from the fresh tissue weight. This, however, is another effect of the alteration in the hydration factor of the stem solids; in terms of concentration in the organic solids, there was a decrease.

Change in the form of nitrogen in the culture solution is not the only condition that produces such an effect. Potassium deficiency also diminished both the quantity and the concentration of the organic acids in the leaf tissue as is shown by the points for these plants plotted in the figure. More complete investigation of this matter is clearly required.

Figure 11 shows the quantities of the individual organic acids in the
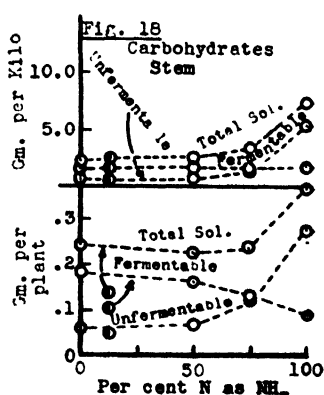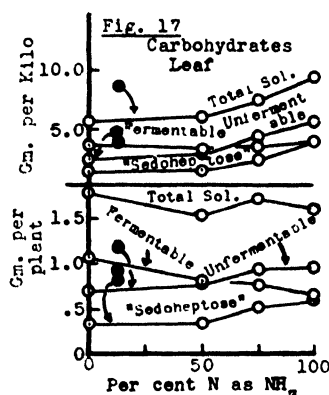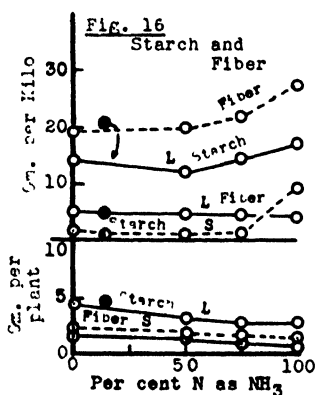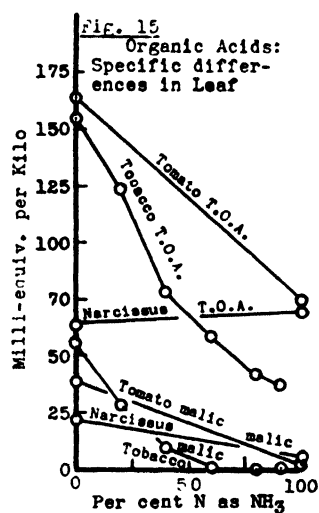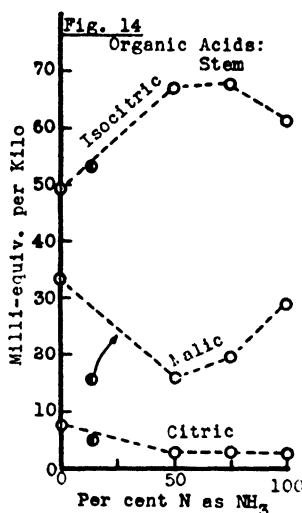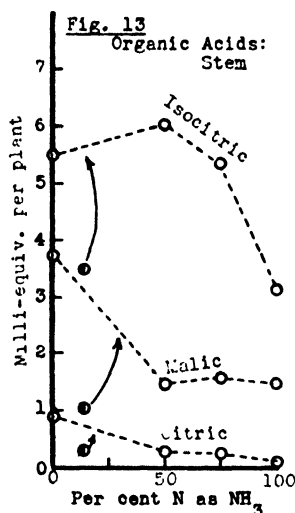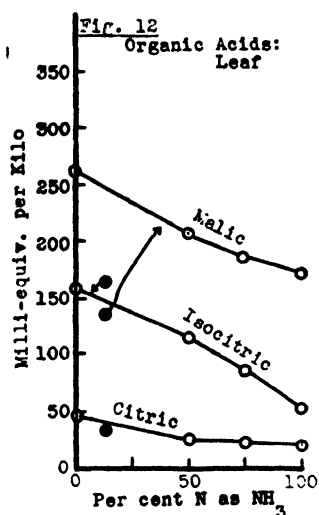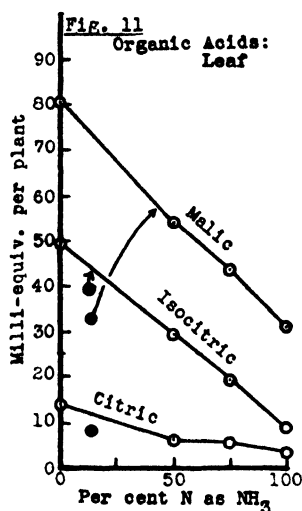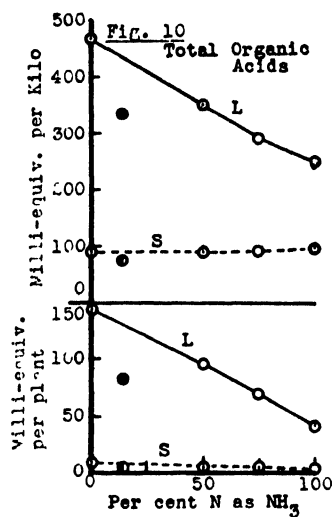
FIGS. 10–18. Analytical data for organic acids, carbohydrates, and crude fiber.

leaf. It will be noted that, in this series of plants, the malic acid considerably exceeded the isocitric acid, a condition unlike that encountered in the plants described in the previous paper of this series (8). The difference arose as a result of the time of day at which the plants were harvested. The present plants were collected at 8:00 A.M. (Standard Time), at which time the leaves were still enriched in organic acids and the deacidification process had not proceeded very far. The plants described in the previous paper, on the other hand, were collected a few minutes past noon on sunny days, the time and conditions being selected so that the deacidification process had become quite extensive although it was probably not complete. Inasmuch as malic acid undergoes major changes in quantity during the day, the reason for the difference in the composition of the plants employed in the two experiments is clear.

The effect of increase in proportion of ammonia in the culture solution upon the quantity of malic acid in the leaves is striking. If it can be granted that the several groups of plants were harvested at exactly the same stage in the deacidification process (and the smoothness of the curves suggests that the precautions taken to achieve this were moderately successful), there seems little doubt that malic acid is in some way concerned with metabolism of nitrate. The functions of this acid in the plant are doubtless complex; if present-day views of the chemical mechanisms concerned with respiration are correct (1, 15), malic acid forms one of the members of the group of organic acids which undergoes enzymatic transformations, the net result of which is the absorption of oxygen and elimination of carbon dioxide. These transformations are conceived to be arranged in a cycle (3, 5) so that, as malic acid is used it is also renewed, the total concentration present supposedly remaining essentially unchanged. This sequence of reactions must go on not only in the nitrate plants but also in the ammonia plants. Accordingly, if it be assumed that the respiration rate per plant did not vary greatly over the series (an assumption that is safe only as a first and very rough approximation), it follows that the quantity of malic acid in the 100% ammonia plants represents the quantity required for respiration alone. What, then, is the function of the considerably larger quantity of malic acid in the nitrate plants? The sequential change in quantity over the series, as the proportion of nitrate in the culture solution diminished, strongly suggests that malic acid is involved in the reactions whereby nitrate ion is reduced. If this is so, it would appear that somewhat more than one-half (the figures suggest five-eighths) of the malic acid present may be so concerned.

Even this supposition, however, does not necessarily exhaust the possibilities. The Bryophyllum plant is a species which undergoes extensive diurnal change in malic acid content, and there is no reason to assume that the fluctuations are in any way concerned with the reduction of nitrate, although they may be concerned with the respiration. Nevertheless, the possibility should not be overlooked that there is a third and still unknown

function of malic acid, this function being one that is especially pronounced in crassulacean plants.

Probably the most striking feature of figure 11 is the fact that the diminution of malic acid is accompanied by a corresponding diminution in isocitric acid content. Over the greater part of their course, the two curves are parallel straight lines within the limits of error of measurement. Thus, the situation that led to a less extensive synthesis of malic acid had an equal effect upon the quantities of isocitric acid that were formed in the leaf tissue.

A metabolic relationship between these two acids is to be anticipated; both are members of the so-called tricarboxylic acid cycle of KREBS (3) and are known to be concerned in the respiration of animal tissues. However, that both should be equally affected by the replacement of nitrate in the culture solution by ammonia implies that both in some way enter into the reactions whereby nitrate is reduced. The observation foreshadows the erection of an hypothesis of the mechanism for reduction of nitrate in plant tissues. This hypothesis will involve a sequence of enzymatic transformations of organic acids, possibly a series arranged in a cycle, and it seems evident that two of the substances concerned in this series, at least in the Bryophyllum plant, will be malic and isocitric acids.

Citric acid also was diminished in quantity in the leaf tissue by the change in culture solutions. The change involved a drop from 14 m.e. to 3.5 m.e. of acid per plant, a proportionately large change, greater even than the change in malic acid which dropped from 80 m.e. to 31 m.e. The metabolism of citric acid is thus also probably involved in the processes that bring about reduction of nitrate, and this substance may be a third member of the interrelated series of organic acids concerned.

That malic and citric acids are intimately related in metabolism in leaves is well known. For example, conversion of malic into citric acid, or at least the replacement of malic by citric acid during the culture of tobacco leaves in darkness has been demonstrated (9). The enzymatic relationship of isocitric to citric acid, presumably via aconitic acid, is also well known in animal tissues (4, 6). Accordingly, the present observations are suggestive of mechanisms that will doubtless be clarified when the enzyme systems present in leaves are finally understood.

Figure 12 shows the changes in concentration of organic acids of the leaf; the data, calculated in this way, confirm the conclusions already drawn from the data plotted in figure 11. Changes both in quantity and in concentration were essentially similar. The effect of potassium deficiency upon the organic acids is shown in figures 11 and 12. Both the quantity and the concentration of malic acid were greatly depressed under these circumstances although the effect upon the isocitric acid was small. Potassium deficiency thus interfered with metabolic processes that involved malic acid proportionately far more than it did with isocitric acid, and the citric acid was also only slightly affected. Interpretation of this observation must await further study.

Figure 13 shows the effect of composition of the culture solution on the organic acids of the stem tissue.   Malic acid was depressed in quantity up to the 50% level of ammonia nutrition but was not changed further.   Isocitric acid, on the other hand, was not greatly changed in amount at the 50% level, a small increase taking place.   At the highest level of ammonia nutrition, however, isocitric acid dropped to about one-half of the amount present in the nitrate plants.   In the stem tissue, therefore, the close relationship between malic and isocitric acids no longer holds.   Furthermore, in this tissue, isocitric acid is present throughout the series in larger quantities than malic acid.   The actual quantities present were small (the scale of figure 13 is 12½ times greater than that of figure 11) and thus the changes, although proportionately large, were actually small with respect to the amount of substance involved.

Figure 14 shows the concentration changes in the stem.   Malic acid diminished sharply at the 50% level of ammonia nutrition but then increased.   Isocitric acid increased in concentration at the 50% level, maintained this at 75% and then dropped somewhat in the 100% ammonia plants.   Citric acid dropped to less than one-half at the 50% level but then remained essentially constant.

The behavior of organic acids in the stems of potassium-deficient plants is especially interesting.   The quantities of all three acids were much smaller than would correspond to the position of plants on the scale of ammonia nutrition, malic and isocitric acids being particularly low.   Data for concentration show, however, that isocitric acid falls almost exactly in line with data for the ammonia series although malic acid was depressed.   Nevertheless, these changes were small in absolute amount, although they appear large on the scale of the figures; the irregularities in the apparent behavior of the organic acids in stem tissue are thus magnified.

A comparison of results on the effect of ammonia nutrition on organic acids in plants with some of the data from the literature is attempted in figure 15.   The only case in which data are available for the progressive changes resulting from gradual alteration of the form of nitrogen in the culture solution is that of tobacco (16).   Figures for the concentration of total organic acids and for malic acid in the leaves are plotted and show an effect that is, in most respects, exactly comparable with data in figures 10 and 12.   Although the concentrations in the tobacco plants cultured on nitrate alone were much lower than were those for Bryophyllum, the drop was proportionately even greater.   The tobacco leaves grown with 90% of the nitrogen supplied as ammonium ion contained a concentration of organic acid only 24% of that in the nitrate plants.   The corresponding figure for Bryophyllum, although at the 100% level, was 55%.   Malic acid dropped to 2.7% of that present in the nitrate cultured tobacco plant, to 69% in Bryophyllum.   These figures alone are sufficient to suggest that organic acids, particularly malic acid, in Bryophyllum leaves have functions that must fall into categories different from their functions in the tobacco plant although, in both

species, there is no reason to doubt that malic acid is in some way concerned with nitrate reduction. In the present series of Bryophyllum leaf samples, we are dealing with the plant at a high point in its diurnal process of enrichment with acids. It is doubtful that this process has anything to do with the reduction of nitrate. Bryophyllum plants grown with ammonium salts as the sole source of nitrogen likewise exhibit a diurnal variation in acidity (unpublished observations). Accordingly, whatever this phenomenon may mean in the general course of metabolism of the species, it is common to all members of the present series. Failure of the malic acid to decrease to the extremely low level observed in the tobacco plant grown with a high proportion of its nitrogen supplied as ammonium salt is doubtless connected with this fact.

Data calculated from CLARK's study (2) of the tomato plant grown in nitrate or in ammonium salt culture solutions are also shown (fig. 15). Points at the two extremes only are available and these are arbitrarily connected with a straight line. Total organic acids and malic acid behave in a manner much like that of the tobacco plant, as might be expected from the close botanical relationship. Data from a recent study of the narcissus bulb (17), however, show a small increase in the concentration of total organic acids when the bulbs are grown with nitrogen supplied as ammonium salt. This case is fundamentally different; the narcissus bulb possesses sufficient stores of nutriment to support growth of the plant into the flowering stage without outside sources of nitrogen. The supply of either nitrate or ammonium salts in the culture solution does, indeed, promote a more luxuriant growth, but it is not essential and there are many other more subtle chemical effects. Organic acids in the leaves of this species are normally low in concentration and malic acid, being of secondary importance to the group of ''unknown'' organic acids, is not the predominating acid. It was the group of ''unknown'' organic acids that underwent the most marked increase in concentration, the figures being 32.5 m.e. per kilo in the leaves of the bulbs grown in nitrate solution and 51.2 m.e. per kilo in those grown with ammonium salt. Malic acid, on the other hand, followed the general rule; it decreased from 20.2 m.e. to 5.5 m.e. per kilo of fresh weight. Thus, in this species also, malic acid responds to ammonia nutrition as it does in the tobacco and Bryophyllum plants.

One further phase of the organic acid changes in Bryophyllum plants should be discussed. Comparison of the lower part of figure 1 with that of figure 10 shows that the loss· of organic solids from the leaf tissues can, in considerable part, be accounted for in terms of the loss of organic acids. The figures in table V are given to permit a closer comparison of the two. They show the change from the nitrate plants, as the basis of comparison, of the organic acids (computed in grams) and the organic solids. The ratio of the two quantities shows that from 78% to 65% of the change in organic solids in leaves can be accounted for as the loss of organic acids. In the postassum-deficient plants, the figure is 92%. This computation is doubt-

TABLE V

RELATIVE DECREASE IN ORGANIC SOLIDS AND ORGANIC ACIDS OF BRYOPHYLLUM PLANTS AS
NITRATE NITROGEN IN THE CULTURE SOLUTION IS REPLACED BY AMMONIUM
NITROGEN.   (FIGURES ARE DIFFERENCES IN GRAMS FROM PLANTS GROWN
IN CULTURE SOLUTIONS THAT PROVIDED ALL NITROGEN AS NITRATE)

TISSUE	PERCENTAGE OF NITROGEN AS AMMONIUM NITROGEN			POTASSIUM-DEFICIENT*
	50%	75%	100%	
Leaf:				
Decrease, organic solids (grams)	4.51	6.42	10.15	4.54
Decrease, organic acids (grams)	3.52	4.93	6.60	4.18
Ratio, loss of acids to loss of solids	0.78	0.77	0.65	0.92
Stem:				
Decrease, organic solids (grams)	1.16	1.17	1.73	2.54
Decrease, organic acids (grams)	0.147	0.187	0.339	0.340
Ratio, loss of acids to loss of solids	0.13	0.16	0.20	0.13

* Plants with 14% of the nitrogen in the culture solution as ammonium nitrogen and the balance as nitrate.

less an oversimplification of the course of chemical events, particularly in the case of potassium-deficient plants where other factors than the form of nitrogen supplied entered into the alteration in chemical composition. It is clear, however, that the leaves of plants supplied with an increased proportion of ammonium ion in the culture solution contained successively less and less organic substance and that the change in organic acid composition goes far to account for the change. This was not true for the stems; here the change in organic acid content was small both in absolute amount and in relation to the change in organic solids. The metabolic processes of the two tissues are manifestly quite different.

Another method of expressing the change in organic acid content in relation to the changes in other important components of the leaves is shown in table VI. Here, the changes in nitrogen, protein, organic solids and carbohydrates are calculated as percentages of the quantity in the leaves of

TABLE VI

PERCENTAGE CHANGES OF COMPONENTS OF BRYOPHYLLUM LEAF TISSUE BROUGHT ABOUT BY
ALTERATION OF THE FORM OF NITROGEN IN THE CULTURE SOLUTION.   (FIGURES ARE
LOSSES OR GAINS COMPUTED AS PERCENTAGE OF QUANTITY OF THE GIVEN
COMPONENTS IN LEAVES OF THE NITRATE PLANTS)

ORGANIC FRACTIONS	PERCENTAGE OF NITROGEN AS AMMONIUM NITROGEN			POTASSIUM-DEFICIENT
	50%	75%	100%	
				%
Organic acids	− 37.3	− 52.2	− 69.8	− 43.8
Total nitrogen	− 9.3	− 17.2	− 41.5	− 1.8
Protein nitrogen	+ 23.3	+ 7.8	− 29.0	− 10.5
Organic solids	− 16.3	− 23.7	− 37.4	− 13.1
Carbohydrates	− 11.5	− 1.2	− 8.0	+ 18.3

the nitrate plants and compared with the percentage change in the organic acids. Changes of the order of 10% may be assumed to be on the borderline of the limits of accuracy of analytical methods; changes substantially greater than this are significant. It is clear, from the first line of the table, that changes in the organic acids are of great relative importance; change in total nitrogen is fully significant at the 75% and 100% levels of ammonia nutrition, that of the protein at the 50% and the 100% levels, one being an increase, the other a loss. Change in the organic solids is probably significant at all three levels, but that of the carbohydrates may not be save in the potassium-deficient plants where an increase occurred. Organic acids are thus outstanding in the sensitivity of their response to the form of nitrogen in the culture solution.

Carbohydrates

Figure 16 shows changes in starch and crude fiber, the quantities per plant being shown in the lower part and concentration in the fresh tissue in the upper part of the diagram. Starch in the leaf dropped slightly at the 50% level of ammonia nutrition and subsequently remained essentially constant. Starch in the stem was too small to represent on the scale of the diagram; it ranged from 0.2 gm. in the nitrate plants to 0.1 gm. in the 50% and 75% ammonia plants, but was increased to 0.48 gm. in the stems of the 100% ammonia plants. Fiber in both leaf and stem tissue followed straight line curves that sloped only slightly downward with increase in the proportion of ammonia.

The curves for concentration show a slight decrease in starch in leaves at the 50% level followed by a moderate rise. In the stem there was no change except in the 100% ammonia plants, the stems of which were relatively enriched in starch. Fiber in the leaves scarcely changed, but in the stems there was a gradual increase.

Soluble carbohydrates of the leaf are shown in figure 17. It should be remembered that the soluble carbohydrates are, like the organic acids in this species, active metabolites; nevertheless, the curves are relatively smooth. Soluble carbohydrates do not form a large proportion of organic solids in this species; the response to the change in form of nitrogen supplied to the plants was not particularly striking. Total soluble carbohydrates underwent small fluctuations in quantity in the leaves, this being apparently a result of a moderate, progressive increase in unfermentable carbohydrate together with a moderate decrease in fermentable carbohydrate. The data expressed on a concentration basis suggest a continuous increase in total carbohydrates, unfermentable carbohydrates and in the component that is recorded as "sedoheptose."[1] The fermentable carbohydrate, much if not all of which is presumably glucose, diminished slightly in concentration over the series.

The behavior of the soluble carbohydrates in the stem tissue is shown in figure 18. The scale of the lower part of this figure is five times greater than

[1] For explanation of this component, see paper 1 of this series (8).

TABLE VII

COMPOSITION OF THE BRYOPHYLLUM PLANT AS AFFECTED BY THE FORM OF NITROGEN SUPPLIED IN THE CULTURE SOLUTION

ORGANIC FRACTIONS	LEAF					STEM				
	PERCENTAGE OF NITROGEN AS AMMONIUM NITROGEN				POTASSIUM-DEFICIENT	PERCENTAGE OF NITROGEN AS AMMONIUM NITROGEN				POTASSIUM-DEFICIENT
	0%	50%	75%	100%		0%	50%	75%	100%	
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Protein (N×6.25)	2.33	2.87	2.51	1.65	2.08	0.369	0.350	0.337	0.275	0.275
Soluble nitrogenous components (N×10)	2.56	1.15	1.35	1.20	2.90	0.75	1.07	0.88	0.71	0.78
Starch	4.35	3.10	2.84	2.90	4.96	0.203	0.103	0.127	0.480	0.055
Crude fiber	1.56	1.24	1.07	0.772	1.11	2.17	1.80	1.75	1.43	1.35
Ether extract	0.825	0.724	0.678	0.304	0.571	0.097	0.087	0.100	0.091	0.070
Soluble carbohydrates	1.75	1.55	1.73	1.61	2.07	0.241	0.228	0.260	0.373	0.151
Isocitric acid	3.16	1.91	1.28	0.58	2.53	0.352	0.387	0.345	0.202	0.221
Malic acid	5.40	3.60	2.91	2.07	2.24	0.248	0.096	0.106	0.099	0.068
Citric acid	0.907	0.434	0.348	0.228	0.518	0.056	0.018	0.015	0.007	0.018
Oxalic acid	0.008	0.007	0.007	0.006	0.007	0.003	0.012	0.007	0.013	0.013
Total known organic solids (estimated)	22.85	16.58	14.72	11.32	18.98	4.49	4.15	3.93	3.68	3.00
Total organic solids (determined)	27.12	22.61	20.70	16.97	22.58	7.01	5.85	5.84	5.28	4.47
Unknown organic solids	4.27	6.03	5.98	5.65	3.60	2.52	1.70	1.55	1.60	1.47
Unknown as % of total organic solids	15.74	26.7	28.9	33.3	15.9	35.9	29.1	26.5	30.3	32.9
Inorganic solids	3.47	2.44	1.91	1.26	1.94	0.95	0.77	0.58	0.37	0.39

that of figure 17, so the rather small enrichment of the stems of the 100% ammonia plants in total and unfermentable carbohydrates is relatively magnified. The quantity of fermentable carbohydrate decreased but the concentration remained nearly constant. However, the concentration of unfermentable carbohydrate increased.

It will be noted that the behavior of carbohydrates in the potassium-deficient plants is definitely out of line with that of the ammonia series of plants. With the exception of the fermentable carbohydrate, the leaves were relatively enriched. In the stems the unfermentable carbohydrate falls close to the line, but the fermentable carbohydrate and total carbohydrates were depressed.

Influence of ammonia nutrition on composition

Table VII shows estimates of the composition of the ammonia series of plants together with that of the potassium-deficient plants which received 14% of their nitrogen as ammonium ion. The conventions that have been used in calculating the composition have been described in the previous paper of this series (8).

Sums of the estimates of the composition are shown in the eleventh line of the table and are to be compared with the determined organic solids shown in the next line. Differences between the respective sets of figures, shown as unknown organic solids in line thirteen, furnish a measure of the quantity of organic material for which no account can at present be given. These quantities are calculated as percentages of the determined organic solids in the next to last line. The figures show that from one-sixth to one-third of the solids of leaf tissue of the ammonia series of plants consists of material concerning which no qualitative information is available. For the potassium-deficient plants, the unknown fraction is also about one-sixth. These figures are comparable in magnitude with the results of a similar survey of data obtained in the growth experiment with this species.

The accounting for stem tissue in the present series is superior to that of plants described in the previous paper. Only from one-quarter to one-third of the organic solids remain unassigned in contrast to somewhat more than one-half. Whether or not this was a result of the different time of day the plants were collected remains for further study. Nevertheless, the conclusion previously drawn, that much remains to be learned of the qualitative composition of this plant, remains valid.

Summary

It has long been known that *Bryophyllum calycinum* exhibits a pronounced diurnal variation in composition with respect to organic acids, the leaves becoming enriched, particularly in malic acid, during the night but undergoing marked loss of acidity during the day. Furthermore, culture of plants in general upon nitrate-containing solutions has been observed to lead to increase in the content of organic acids as compared to the effect of

culture upon solutions that provide nitrogen in the form of ammonium ions. In view of these facts, analyses have been carried out of the leaf and stem tissues of *B. calycinum* collected in the morning at a time of relatively high acidity, from plants cultured upon a series of solutions in which a constant concentration of nitrogen was furnished. Composition of the culture solutions was varied in steps in the relative proportions with which nitrate ion and ammonium ion were supplied. The extremes were solutions that provided all nitrate ion and all ammonium ion. Results are presented as curves in which composition is plotted against the percentage of ammonium ion in the culture solution. The curves show composition in terms of grams per plant (absolute quantity) and grams per kilo of tissue (concentration).

In the leaves, the organic solids, water, total nitrogen, nitrate nitrogen, organic soluble nitrogen, total organic acids including isocitric, malic and citric acid, starch, crude fiber and fermentable carbohydrate diminished in absolute quantity per plant with increase in the proportion of ammonium ion in the culture solution. Protein nitrogen, ammonia nitrogen, and glutamine increased to a maximum and then decreased. Asparagine decreased and then increased. Unfermentable carbohydrate increased. In the stems, most of the components followed a similar course except that the ammonia nitrogen and glutamine increased continuously.

On a concentration basis, organic solids of the leaves, together with total nitrogen, starch, soluble carbohydrates, unfermentable carbohydrates and "sedoheptose" increased, the protein nitrogen and ammonia nitrogen increased to a maximum and then decreased, the asparagine and organic soluble nitrogen decreased to a minimum and then increased, while the water, nitrate nitrogen and total organic acids including malic, isocitric, and citric acids decreased. In the stems, the concentration of components followed a similar course except that nitrate nitrogen decreased profoundly while ammonia nitrogen, glutamine, and organic soluble nitrogen increased throughout.

B. calycinum follows the general rule that organic acidity of tissue is diminished by culture on ammonium ion as the source of nitrogen. The change falls upon both malic and isocitric acids and affects the quantity of each of these substances present in the leaves equally. Citric acid is affected to a smaller extent with respect to quantity, but the proportional change is even greater.

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EFFECT OF MINERAL DEFICIENCIES UPON THE SYNTHESIS OF RIBOFLAVIN AND ASCORBIC ACID BY THE OAT PLANT[1]

STANLEY A. WATSON AND G. R. NOGGLE[2]

(WITH SIX FIGURES)

The use during recent years of immature cereal grasses such as oats, wheat, rye, and barley has received considerable attention as a protein and vitamin concentrate for both human and animal consumption, since grasses cut just previous to the jointing stage are high in protein and vitamin content. KOHLER (10) studied the effect of the stage of growth on the composition of the grasses and showed that protein, fat, chlorophyll, carotene, riboflavin, ascorbic acid, and thiamine attained a maximum concentration at, or about, the time of the jointing stage. Pantothenic acid and biotin were found to attain a maximum at a later period in the development of the plant while nicotinic acid was present in maximum concentration at an earlier stage.

Considerable work has been done on the influence of nutrient deficiencies on the composition of plants. The published literature has dealt mainly with such plant constituents as proteins and carbohydrates. A great deal of work has been done on the factors concerned with vitamin C content of plants. Most of the workers agree that environmental factors, particularly light intensity, may greatly affect the ascorbic acid content. There is no general agreement, however, as to the influence of mineral nutrients on the ascorbic acid content of plants. Most of the investigations have been done on fruits or leafy vegetables, but little study has been made of the factors influencing the ascorbic acid content of immature cereal grasses. A number of studies have been made on the effect of soil and nutrients on the riboflavin content of cereal grain, but few investigations have been concerned with the riboflavin content of immature cereal grasses.

The work described in this paper was carried out to determine the effect of single nutrient deficiencies on the ascorbic acid and riboflavin content of the immature oat plant.

Experimental procedures

CULTURAL METHODS

Illinois 30–2088 oats, supplied by the Illinois Agricultural Experiment Station, were used in the experiment. The plants were grown in one-gallon

glazed earthenware crocks filled with no. 9 crushed quartz gravel previously washed with hydrochloric acid.   There were twelve pots of plants per treatment, sixteen to twenty plants per pot.   The nutrient solutions were contained in two-gallon, soft-glass bottles which had been given several coats of aluminum paint to prevent the growth of algae.   The nutrient solution was automatically forced up from the bottles into the pots by means of an air pump blowing air into the closed system.   Each bottle of solution was connected to two pots.   The nutrient solutions were pumped into the pots and remained for a twenty-minute interval every six hours.

The oats were planted on March 16, 1942 with about 20 seeds per pot evenly spaced and covered with one-half inch of gravel.   In order to obtain sufficient plant material for analytical study, the plants were grown for the first three weeks in a complete nutrient solution.   When the plants were 21

TABLE I

COMPOSITION OF THE NUTRIENT SOLUTIONS

TREATMENT	KH_2PO_4	$MgSO_4$	$Ca(NO_3)_2$	$(NH_4)_2SO_4$	$CaCl_2$	$MgCl_2$	KCl	$NaNO_3$	NaH_2PO_4	Na_2SO_4
MILLIMOLES PER LITER OF SOLUTION										
Control	2.27	2.09	4.09	0.33						
– K	.	2.09	4.09	0.33					2.27	
– Ca	2.27	2.09		0.33				8.18		
– Mg	2.27		4.09	0.33						2.09
– NO₃	2.27	2.09		†	4.09					
– SO₄*	2.27		4.09	†		2.09				
– PO₄		2.09	4.09	0.33			2.27			

* Iron added as $Fe(NO_3)_2$-citrate. $FeSo_4$-citrate added to all others.
† Ammonium salts omitted from these cultures.

days old, the deficient solutions replaced the complete nutrient solutions. At this time the plants had three fully expanded leaves and a fourth leaf was emerging.   The plants were harvested April 28, 42 days after seeding. The plants had an average of seven well-developed leaves and all had at least one joint; some had two joints.   The weather was clear and sunny on the day of harvest and on the two preceding days.

The complete solution of macronutrients as shown in table I was that of SHIVE and ROBBINS (17).   The micronutrients were supplied in the form of the solution described by HOAGLAND and ARNON (6).   Boron and manganese were present at a level of 0.5 p.p.m.   Iron was supplied in the form of ferrous citrate prepared by dissolving 5.0 gm. of $FeSO_4 \cdot 7H_2O$ and 2.5 gm. of citric acid in distilled water and diluting to 1 liter.   Ferrous nitrate in place of ferrous sulfate was used for the sulfur-deficient series.   The iron content of the nutrient solution was checked regularly by the thiocyanate colorimetric method given in Standard Methods of Water Analysis (21).

It was found necessary to add 5.0 ml. of the iron solution to each bottle every three or four days to keep the concentration in the range of 0.05 to 0.08 p.p.m.

The pH of the solutions was checked every third day and when necessary the pH was adjusted with 0.25 N sulfuric acid or NaOH to a pH of approximately 5.5. In the sulfur-deficient series, HCl was used for the pH adjustment. Ammonium sulfate was added to the nutrient solution to stabilize the pH of the solutions as the ions were withdrawn by the plant. The amount of ammonium sulfate solution was added to give a NO_3/NH_4 ratio of about 80/20 to 90/10, which, according to TRELEASE and TRELEASE (22), should hold the solutions at a pH of about 5.1. No ammonium sulfate was added to the sulfur and nitrate-deficient series.

The nutrient solutions were completely renewed every ten days during the first three weeks and then every seven days during the rest of the growing period. When the change-over was made from full nutrient solution to the solutions lacking the specified elements, the pots were flushed several times with distilled water.

Some trouble was experienced in regard to iron chlorosis. In addition to the iron added regularly to the nutrient solution, treatments were given on April 12, 17, and 25 by shutting off the regular iron solution and allowing the roots to stand in contact with an iron solution for six hours. The iron solution consisted of 25 ml. of the stock iron solution diluted with distilled water in the pot so that the surface of the gravel was covered.

At harvest time, two plants per pot from each treatment were cut for the analysis of ascorbic acid, the plants separated into leaves and "stems," composited separately from each treatment, and analyzed immediately. The pots were then placed in a tub of water and the gravel was carefully washed from the roots. The last bits of gravel were removed by hand. The tops were severed from the roots at the crown, the water blotted from the tissue, and fresh weight determined on both the tops and the roots. The tops were further separated into leaf blades and "stems" by clipping the leaves next to the ligule. The "stem" thus consisted of the true stem plus the leaf sheath. Dead leaves were discarded. Hereafter, the leaf blades will be referred to as "the leaves." The weights per plant were determined by dividing the weight of the composited sample from the twelve pots of each treatment by the number of plants obtained from each treatment. Each sample contained the respective parts from about 175 plants.

The plant parts were spread out on paper and dried rapidly in an oven ventilated by a stream of air at 70° C. The plant material dried in four hours. The dry weight was determined and the tissue was ground in a Wiley mill to pass a 60-mesh screen. The plant material was stored in cardboard boxes until analyses could be made.

ANALYTICAL METHODS

Total nitrogen was determined by a micro-Kjeldahl method of PEPKOWITZ and SHIVE (15) using metallic selenium as the catalyst.

The ash components were determined on a wet-ashed sample prepared and analyzed as described by NOGGLE (13). One gram of air-dried grass was placed in a flat-bottomed porcelain crucible and dried at 105° C. for eight hours and then reweighed to obtain the oven-dry weight. All analytical results are reported on the basis of oven-dry weight.

Ascorbic acid was determined by a titrimetric procedure. Reagents consisted of a fresh aqueous acid solution containing 2% metaphosphoric acid, 3% trichloracetic acid, and a dye solution. The standard dye solution contained 1 gm. of sodium 2,6-dichlorobenzenoneindophenol dissolved in 1 liter of a 1% acetic acid solution of dioxane. The dioxane was freshly distilled before use. The dye solution was standardized against 1 ml. of freshly prepared ascorbic acid solution, containing 0.2 mg. ascorbic acid (10 mgm. ascorbic acid in 50 ml. of the acid solution). To determine ascorbic acid, a 1-gm. sample of fresh grass was triturated with 25 ml. of the acid solution and several grams of pure Ottawa sand in a mortar. The resulting mixture was filtered through No. 202 Reeve Angel filter paper and 1 ml. titrated with the standard dye solution. The titration was completed in less than one minute.

Riboflavin was determined as described in the original microbiological method of SNELL and STRONG (20). The riboflavin was released by autoclaving with distilled water, and assayed in triplicate at three different levels. Thus each sample was subjected to nine determinations. Very little drift in the values was obtained between different riboflavin levels. Very good agreement of values between the various composited samples was obtained. The standard deviation of the values for each type of tissue was: leaves ± 0.324, stems ± 0.251, and roots ± 0.225 gamma per gram. These statistics were calculated from the sums of squares of riboflavin values pooled for each type of tissue (19).

Results and discussion

DESCRIPTION OF PLANTS AT TIME OF HARVEST

The control plants grown in complete nutrient solution for the entire growth period were completely normal as regards outward appearance. The plants had an average of seven leaves. Almost all showed the first joint and many had a second joint.

The potassium-deficient plants showed no noticeable symptoms. The plants appeared to be normal green in color with stems possibly weaker than those of the controls. Tillering was not affected and the roots appeared to be normal.

The calcium-deficient plants exhibited striking symptoms. The youngest leaves were chlorotic, spindling, gelatinous, and stunted. The older leaves were very dark green, coarse, stiff, and erect; the color was much darker than that of the controls. The stems were short, thick, and stiff with short internodes. Most of the plants had at least one well-developed tiller and many small ones that did not develop. The small tillers had the

same appearance as the young leaves. The roots appeared to be normal with the exception of more numerous coarse secondary roots.

The nitrate-deficient plants were the smallest of all the series. The leaves were very erect, standing up like packages of bristles, but were stunted in height and width; the color was a uniform pale green with no mottling. The first and second leaves were dead and slightly red in color; the dead tips of younger leaves were also red. The stems were short and spindling and exhibited less jointing than the controls.

The sulfur-deficient plants showed no particular symptoms except that they appeared to be slightly smaller than the controls. The plants low in phosphate showed no particular symptoms. The leaves were dark green although not as dark as in the – Ca series.

GROWTH AND MINERAL CONTENT

The results of the mineral analyses of the various plant fractions grown on the deficient solutions are given in table II. The data indicate that the deficient solutions have produced plants whose tissues contain lowered

TABLE II

COMPOSITION AND DRY WEIGHT OF THE ORGANS OF OAT PLANTS GROWN ON THE MINERAL SOLUTIONS GIVEN IN TABLE I. VALUES CALCULATED ON AN OVEN-DRY WEIGHT BASIS

NUTRIENT TREATMENT	DRY WEIGHT	ASCORBIC ACID	RIBO-FLAVIN	K	Ca	Mg	N	P
	$g./pl.$	$mg. \%$	$\gamma/g.$	$\%$	$\%$	$\%$	$\%$	$\%$
LEAVES								
Control	0.267	440	21.5	6.67	0.89	0.39	5.61	0.29
– K	0.243	635	20.7	1.82	0.97	0.56	5.38	0.54
– Ca	0.248	356	17.5	5.01	0.23	0.41	4.79	0.54
– Mg	0.229	814	14.8	6.67	0.81	0.07	5.00	0.35
– NO₃	0.119	369	10.6	5.76	0.79	0.29	2.07	0.61
– SO₄	0.193	533	19.3	6.16	1.17	0.41	4.88	0.26
– PO₄	0.205	488	18.7	6.82	0.89	0.39	5.18	0.09
STEMS								
Control	0.133	338	5.3	10.11	0.69	0.36	4.67	0.27
– K	0.125	227	4.8	2.50	0.79	0.46	4.70	0.30
– Ca	0.169	188	4.6	6.40	0.11	0.34	4.55	0.30
– Mg	0.128	490	4.1	8.90	0.85	0.07	4.75	0.29
– NO₃	0.103	148	3.5	6.15	0.57	0.28	1.09	0.19
– SO₄	0.173	214	4.9	9.75	0.89	0.39	3.92	0.23
– PO₄	0.150	224	5.0	9.95	0.76	0.39	3.89	0.11
ROOTS								
Control	0.099	....	6.6	4.83	1.23	0.31	3.67	1.06
– K	0.095	....	6.7	4.79	1.55	0.35	3.69	0.46
– Ca	0.077	....	6.8	4.50	0.32	0.37	3.82	0.83
– Mg	0.100	....	6.4	4.66	1.36	0.19	3.74	0.47
– NO₄	0.088	....	4.2	4.58	0.48	0.31	1.04	0.36
– SO₄	0.101	....	6.7	4.84	2.10	0.33	3.24	1.26
– PO₄	0.082	....	6.5	5.92	0.56	0.26	3.50	0.17

amounts of the element omitted from the nutrient solutions.   Sulfur was
not determined.   Iron was determined but showed ħo important variations.
All plants apparently contained adequate quantities of iron at time of
harvest.

Figure 1 shows that all of the nutrient deficiencies resulted in a fresh
weight production below that of the control series.   In the leaves and stems,
deficiencies of Mg, K, PO_4, SO_4, and Ca depressed fresh weight production
similarly.   The $-NO_3$ series resulted in a fresh weight production less than
one half that of the control.   In the roots the $-Ca$ and $-NO_3$ series gave
the greatest reduction in fresh weight production.   When the total plant
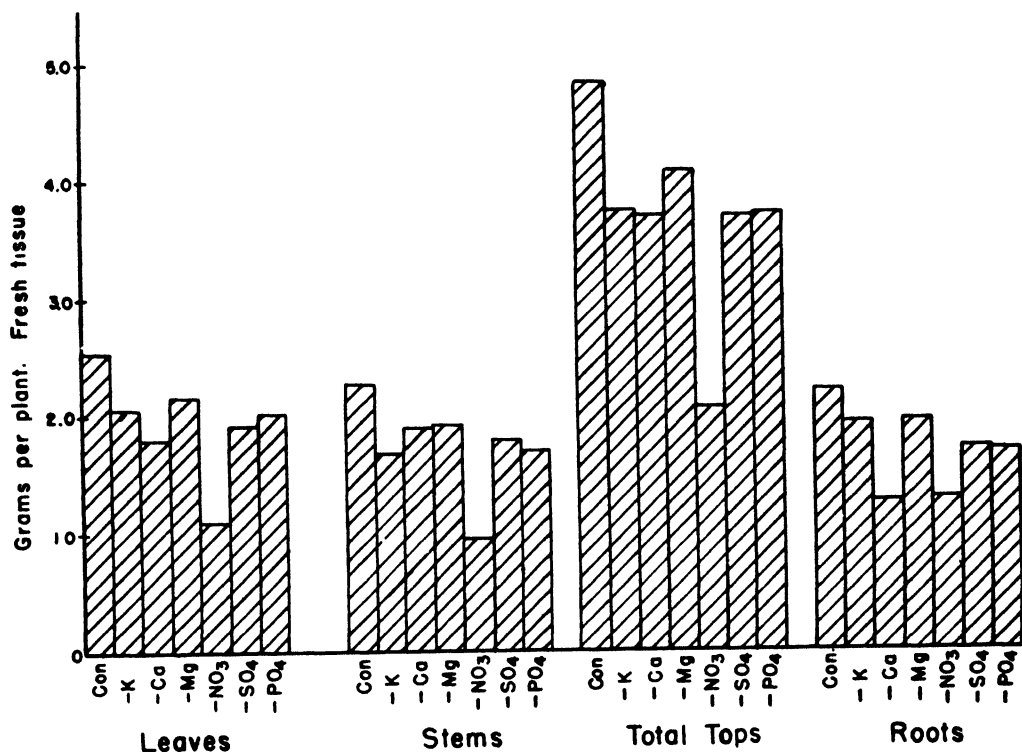
FIG. 1.   Effect of mineral deficiencies upon the growth of leaves, stems, total tops,
and roots of immature oat plants.   Fresh weight of organs per plant.

was considered, the fresh weight production was as follows: Control $>$
$-Mg > -K > -SO_4 > -PO_4 > -Ca > -NO_3$.

The dry weight productions of the various series are compared in figure
2.   In the leaves, the $-Ca$, $-K$, and $-Mg$ series gave dry weight production
not much below that of the control.   The $-PO_4$ and $-SO_4$ series produced
somewhat less dry matter while the $-NO_3$ series produced the lowest amount
of dry matter.   In the stems, the $-SO_4$, $-Ca$, and $-PO_4$ series gave a greater
dry weight production than the control.   There was not much difference in
the production of dry matter in the roots except that the $-NO_3$, $-PO_4$, and
$-Ca$ series were slightly less than the controls.   The total dry weight of the
plants was as follows: Control $> -Ca > -SO_4 > -K > -Mg > -PO_4 >$
$-NO_3$.
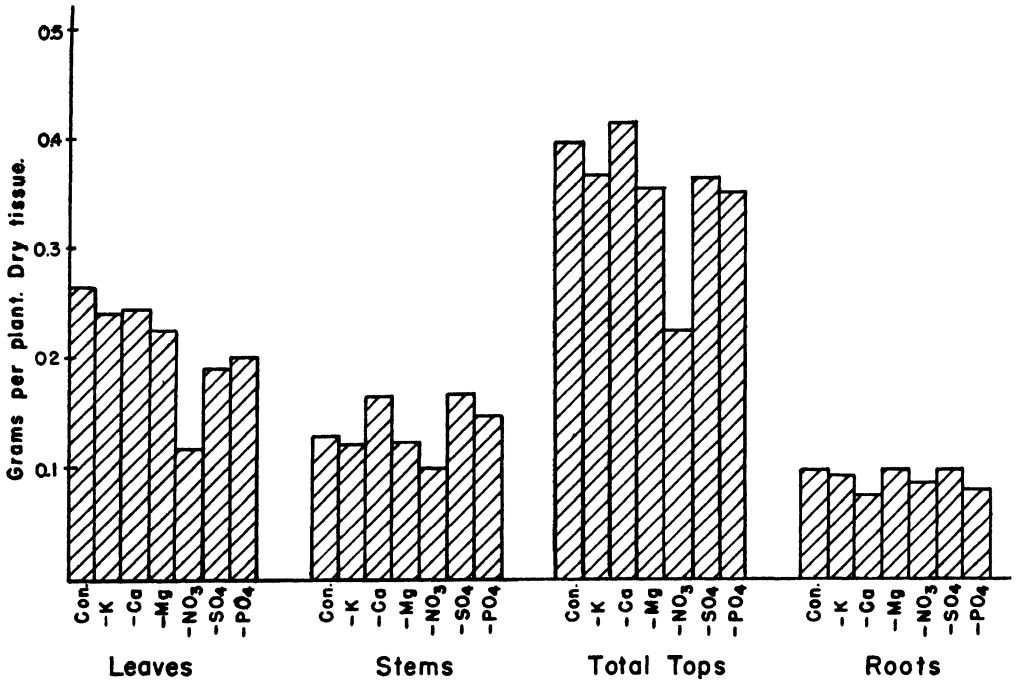
FIG. 2. Effect of mineral deficiencies upon the growth of leaves, stems, total tops, and roots of immature oat plant. Dry weight of organs per plant.

ASCORBIC ACID DATA

The ascorbic acid concentration of the fresh plant tissue is shown in figure 3 (left). The − Mg and − K solutions produced plants whose leaves
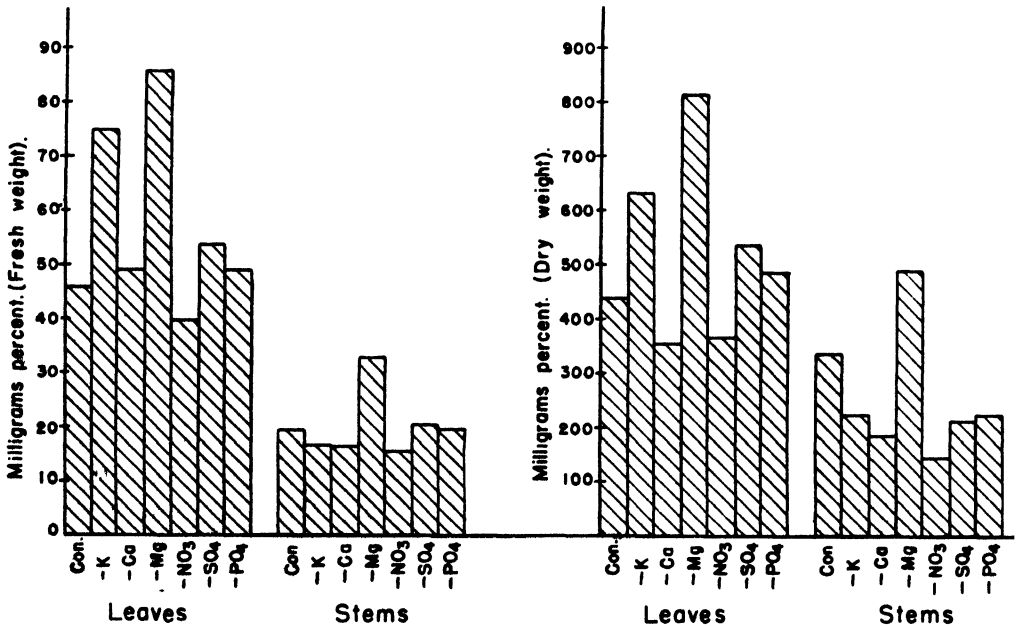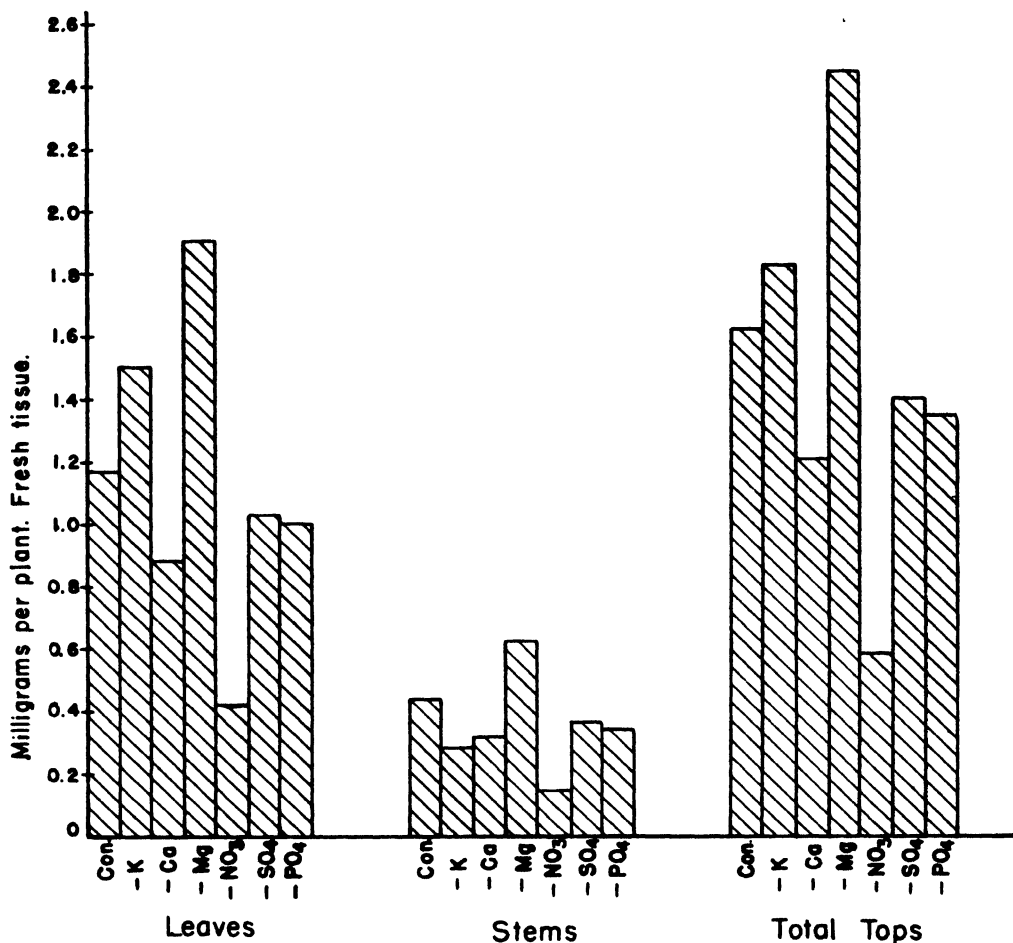

FIG. 3. Effect of mineral deficiencies upon the ascorbic acid content of leaves, stems, and total tops of immature oat plants. Left: milligram percentage in fresh tissue; right: milligram percentage in dry tissue.

contained considerably greater amounts of ascorbic acid than the control plants. The $-SO_4$, $-PO_4$, and $-Ca$ leaves also contained slightly greater ascorbic acid than the control plants. The $-NO_3$ leaves contained less ascorbic acid than the control plants. The stems of the $-Mg$ contained about double the ascorbic acid concentration of the control series. When calculated on the basis of dry weight, (fig. 3, right) the ascorbic acid concentration of the leaves was substantially the same as noted in the fresh tissue;

Fig. 4. Effect of mineral deficiencies upon the ascorbic acid content of leaves, stems, and total tops of immature oat plants. Milligrams ascorbic acid per plant organ; fresh tissue.

i.e., $-Mg > -K > -SO_4 > -PO_4 >$ Control $> -NO_3 > -Ca$. In the stem tissue on a dry weight basis (fig. 3, right), however, only the $-Mg$ treatment resulted in a higher ascorbic acid concentration than the controls while all other treatments resulted in lower ascorbic acid concentration than the controls. Ascorbic acid content expressed as milligrams per fresh plant or plant part (fig. 4) shows the same trends as noted above except that in the cases of the $-Ca$ and $-NO_3$ plants the amount of ascorbic acid per plant

is low owing to the marked lowering of fresh weight production with these treatments.

The effect of magnesium and potassium deficiency on ascorbic acid synthesis in leaves suggested possible relationships between the concentration of these mineral elements in the leaves (table II) and the vitamin concentration. Simple correlation coefficients relating these factors were calculated (19), the results of which are shown in the first column of table III. The correlation of ascorbic acid (mg.% dry wt.) with percentage magnesium and potassium was in both cases negative and very low but scatter diagrams showed pronounced negative trends. The suggestion was made that the true correlations were being masked by the competing effects of these two elements on ascorbic acid synthesis. Under such conditions the true correlations may be estimated by use of the method of partial correlations (11).

TABLE III

SIMPLE AND PARTIAL CORRELATION COEFFICIENTS RELATING ASCORBIC ACID AND RIBOFLAVIN
CONCENTRATION OF LEAVES OF OAT PLANTS TO THE CONCENTRATION OF SEVERAL
MINERAL ELEMENTS IN THE LEAVES

SIMPLE CORRELATION COEFFICIENTS (ZERO ORDER)		PARTIAL CORRELATION COEFFICIENTS (FIRST ORDER)	
FUNCTION†	COEFFICIENT	FUNCTION†	COEFFICIENTS
r_{AM}	− 0.439	$r_{AM \cdot K}$	− 0.653
r_{AK}	− 0.0975	$r_{AK \cdot M}$	− 0.547
r_{KM}	− 0.639		
r_{RN}	+ 0.890**	$r_{RN \cdot M}$	+ 0.780
r_{RM}	+ 0.604	$r_{RM \cdot N}$	− 0.485
r_{NM}	+ 0.819*		

* Significant at the 5% level.
** Significant at the 1% level.
† Symbols of functions are: A = ascorbic acid; K = potassium; M = magnesium; R = riboflavin; N = nitrogen. For partials, $R_{AM \cdot K}$ represents the partial correlation coefficient relating A to M when K is held constant, etc.

This type of analysis enables one to determine the correlations between two variables, independently of the variation caused by the other factor or factors under consideration. In the second column of table III are recorded the coefficients of partial correlation. Neither partial is significant at odds of 19 : 1 (5% level), but the increase in the partial correlation coefficient over the simple coefficient is large in both cases and is in the expected direction.

In view of the above differences in correlation coefficients and the large stimulation in ascorbic acid synthesis in the plants grown on the − K and − Mg solutions, the influence of potassium and magnesium is probably real and should be further investigated. It appears that low concentrations of K and Mg in the leaves stimulate ascorbic acid synthesis while high concentrations retard synthesis. The reverse conclusion, that the presence of high concentrations of K and Mg cause more rapid destruction of ascorbic acid, must also be considered but does not appear to be the most likely explanation.

We should point out that in calculation of correlation coefficients in this paper only seven values were available; i.e., the seven pooled samples from each nutrient treatment. The authors realize that when coefficients of correlation or partial correlation are based on a small number of observations one point in error may cause the coefficient to be higher or lower than the true value. An additional fact which lends added significance to the reported values is that each was obtained from a fairly large, representative composited sample. Each sample used for ascorbic acid analysis was composed of the leaves and stems of 24 plants while all other analyses were made on samples composed of parts of about 175 plants. These samples therefore represent a fairly accurate mean for each treatment.

There is no unanimity among workers as to the influence of fertilizer treatments on the ascorbic acid content of plants. MAYNARD and BEESON (12) reviewed the literature pertinent to ascorbic acid and soil fertility and said: "It seems justifiable to conclude that the accumulation of ascorbic acid in plants is a characteristic of species and variety and that this genetic influence may overwhelm any differences due to climate, soil, or fertilization. Of climatic factors, light seems to have the preponderating influence. The effect of fertilizers is relatively small and varies with the species of plant. It seems that, provided the plant will develop upon the nourishment supplied to it, the ascorbic acid content is not much altered."

In the present investigation the − K and − Mg treatments gave large increases in the ascorbic acid concentration of oat leaves. BERNSTEIN, HAMNER, and PARKS (2) noted that turnip greens grown in sand cultures deficient in sulfate, nitrate, or potassium contained significantly lower ascorbic acid values than the other treatments. With phosphorus and magnesium deficiencies there was little effect on the ascorbic acid content of the turnip greens. REDER, ASCHAM, and EHEART (16) found that potassium fertilization produced a decrease in ascorbic acid content of field-grown turnip greens. SIDERIS and YOUNG (18) grew pineapple plants in low-K cultures and found that the ascorbic acid content depended on the form of nitrogen present. If nitrate nitrogen was present, the low-K series gave high ascorbic acid values; if ammonium nitrogen was present the low-K series gave smaller ascorbic acid values. BALKS and POMMER (1) observed that a K and Mg deficiency generally caused increases in ascorbic acid over that of the control. IJDO (8) found that with spinach a deficiency of potassium resulted in an ascorbic acid content above that of the control, provided the nitrogen supply was adequate. FERRES and BROWN (4) have recently studied the relationship between yield, ascorbic acid, and mineral fertilization in several legumes and leafy vegetables. Using soils known to be deficient in one or more of the elements K, Cu, Zn, Mn, B, and Mo, addition of zinc alone produced significant increases in ascorbic acid concentration. They also found that ". . . increases in ascorbic acid concentration due to application of zinc were in all cases associated with an early growth stage."

WITTWER, SCHROEDER, and ALBRECHT (**23**) pointed out that a considerable body of experimental evidence indicated that there was an inverse relationship between the nitrogen application and ascorbic acid content. Their own work showed that by increasing the application of nitrogen fertilizer the yield of dry matter of Swiss chard and spinach was increased while the concentration of ascorbic acid decreased. IJDO (**8**) concluded that a nitrogen deficiency affected the ascorbic acid content of grass only to a small extent even though the nitrogen content of the tissue was very low. ISGUR and FELLERS (**9**) found that a high level of nitrogen supply gave a high ascorbic acid content. In the present investigation the $-NO_3$ series gave a lower ascorbic acid concentration than did the control series.

Many workers have pointed out that there appears to be a relationship between ascorbic acid and chlorophyll content of plant tissue, but it is interesting to note that, of the two treatments which were highest in ascorbic acid, $-Mg$ and $-K$, the former was strongly chlorotic while the latter appeared normal in color.

RIBOFLAVIN DATA

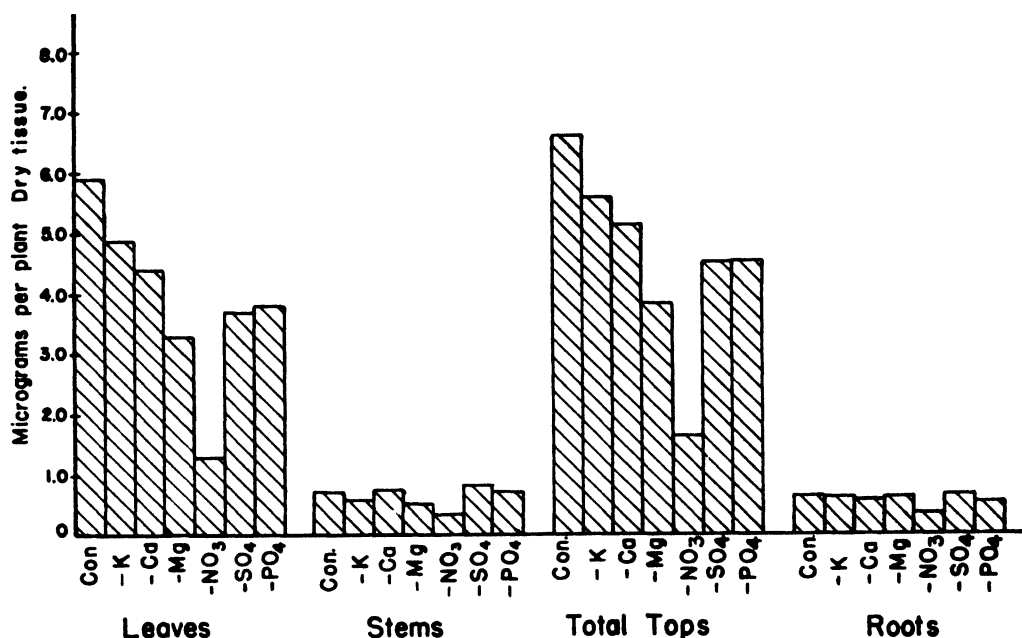The riboflavin concentration of the plant tissue is shown in figure 5. All
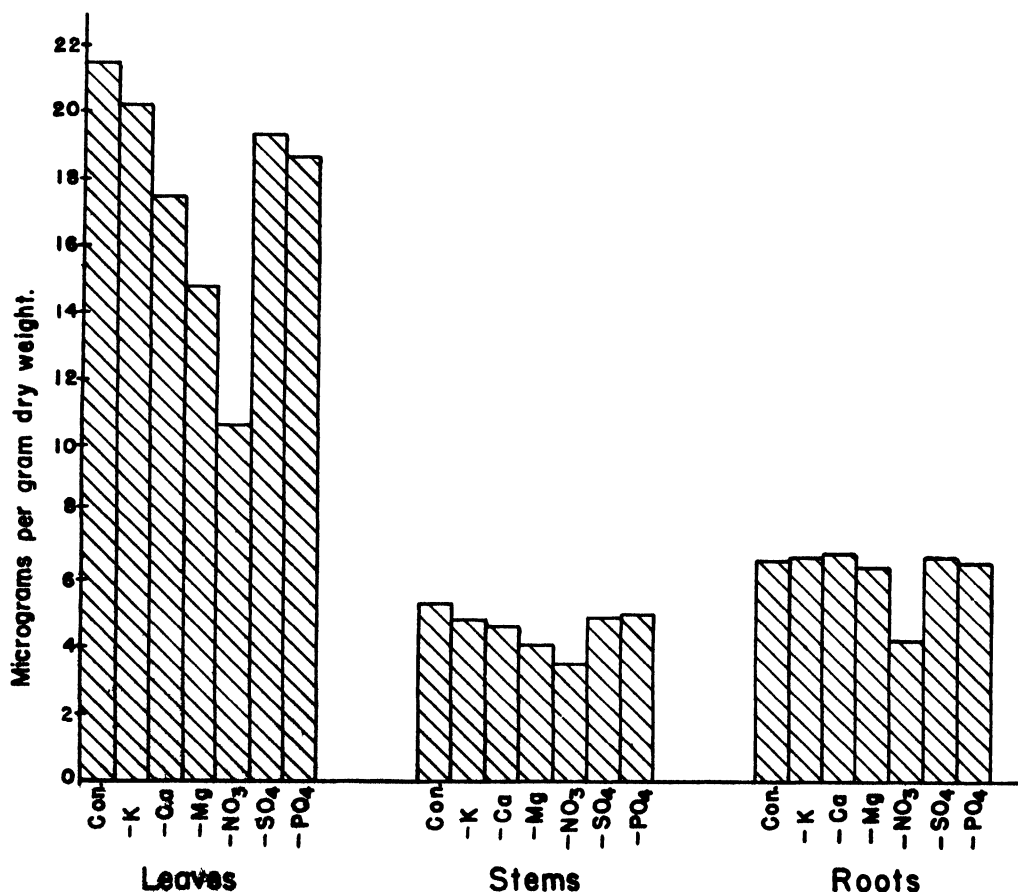
FIG. 5. Effect of mineral deficiencies on the riboflavin content of leaves, stem, total tops, and roots of immature oat plants. Gamma per gram dry weight.

treatments decreased the riboflavin concentration of the leaf tissue, as compared with the control, in the following order: Control $>$ $-$ K $>$ $-$ SO$_4$ $>$ $-$ PO$_4$ $>$ $-$ Ca $>$ $-$ Mg $>$ $-$ NO$_3$. In the stem tissue all deficient solutions resulted in a lower riboflavin concentration than the control. As in the leaf tissue, the $-$ Ca, $-$ Mg, $-$ NO$_3$ series gave the lowest riboflavin concentration in the stems. The $-$ NO$_3$ series alone resulted in a significantly lower riboflavin concentration in the roots than did the control.

In figure 6 the riboflavin content of the plants is expressed on a per plant or plant part basis. In the leaves the trends are the same as was observed in figure 5 but the differences are exaggerated. This is because those treatments which resulted in a lowered riboflavin concentration also resulted in

Fig. 6. Effect of mineral deficiencies on the riboflavin content of leaves, stem, total tops, and roots of immature oat plants. Gamma per plant organ; dry weight basis.

lowered dry weight production. The correlation coefficients (11, 19) relating the growth of the leaves (dry weight per plant) with riboflavin concentration in gamma per gram for the various treatments is $+ 0.743$ and for the growth of stems the correlation with riboflavin is $+ 0.771$. These coefficients are significant at the 5% level (required, r = 0.754) but the correlation of growth of roots with riboflavin ($+ 0.593$) is not significant. Calculations were based on the seven composited samples of each type of tissue. This direct relationship between growth of leaf and stem and riboflavin concentration may indicate a direct dependence of growth of plant tissue upon synthesis of adequate amounts of riboflavin. Such a relationship has not been previously noted for green plants but has been known for many years in animal nutrition. In fact, the basis of the rat assay method for riboflavin is the direct relationship between the growth of rats and their riboflavin intake. However, in the case of green plants where riboflavin is synthe-

sized, and especially in the plants used in the present experiment where the whole metabolic balance is upset by the mineral deficiencies, the relationship may be much more complex than in animals. In the plants described in this paper no important differences in morphological development were observed.

The conditions affecting the riboflavin content of plants has not been extensively studied. The majority of the investigations have been done on cereals but have been concerned with a mature grain or products of the grain such as flour. MAYNARD and BEESON (12) reviewed the work regarding the effect of fertilizers on the riboflavin content of plants. The various studies did not show any marked differences in the riboflavin content due to environmental factors. Fertilizer experiments performed by HOLMES and CROWLEY (7) with lettuce showed complete lack of correlation of riboflavin content with the Ca, P, Mg, or Fe contents of the fresh leaves. A deficiency of boron and manganese in tomatoes and beets was shown by GUM, BROWN, and BURRELL (5) to result in consistently lower riboflavin content of all parts analyzed as compared with controls. The present study reveals that the riboflavin concentration of the immature oat leaves and stems is dependent upon an adequate nitrogen supply. All of the deficient treatments gave lower riboflavin concentrations than the control but the $-NO_3$ treatment gave the lowest value. With the exception of $-NO_3$, the roots did not seem to be adversely affected by any nutrient deficiency.

To bring out more clearly the role of nitrogen and magnesium in the synthesis of riboflavin, correlation coefficients were calculated to test the relationship between the riboflavin concentration and the per cent nitrogen and magnesium in the leaves. The results are given in table III. The highly significant positive value of the simple correlation coefficient indicates that the amount of riboflavin formed in the leaves appears to be directly dependent on the amount of nitrogen absorbed. The importance of this relationship to protein synthesis will be discussed in a forthcoming publication (14). The magnesium concentration of the leaves was positively correlated with riboflavin but was not significant at the 5% level. Calculation of partial correlation coefficients showed that there was probably no mutual influence of nitrogen and magnesium on riboflavin synthesis since in both cases the partial coefficient was lower than the simple coefficient. The low riboflavin concentration found in the plants grown on the magnesium-deficient solution was apparently a result of a low supply of nitrogen since correlation analysis showed that magnesium and nitrogen concentrations in the leaves varied together.

The role of riboflavin as the prosthetic group for several enzymes concerned with cell respiration and other related processes (yellow enzymes, d-amino and 1-amino acid oxidases, etc.) would suggest that for normal metabolic activity this substance must be present in the cells in optimum quantities. For this reason the close relationship observed between growth of the leaves and stems and their riboflavin concentration is not surprising. One would suspect that the effect of mineral deficiencies might cause a cur-

tailment of the synthesis of riboflavin (and other necessary enzymes and hormones) which would in turn retard growth.

The leaf blade of the immature plant is generally considered to be the center of greatest metabolic activity and it is probable that the high concentration of riboflavin in the leaves (fig. 5) reflects the higher metabolic activity of these organs as compared with the "stem" and roots. Reference to figure 6 shows that of the total amount of riboflavin in the normal plant (control series), the leaves contained 4.3 times as much as the rest of the plant or 9 times as much as the "stems" (leaf sheath plus true stem) or roots alone. With the plants grown in the deficient cultures, the ratio of riboflavin in the leaves to the rest of the plant is reduced and reaches a low of 1.9:1 in the nitrate-deficient plants. The roots of the oat plant probably synthesize their own riboflavin (3). If the roots were dependent upon the leaves for their entire supply of riboflavin, one would expect a lower concentration of riboflavin in the roots of the deficient plants than was encountered.

It should be emphasized that the results reported in this paper are from a single experiment during one part of the growing season and are exploratory in nature. It has been pointed out that climatic conditions are very important in influencing the ascorbic acid concentration of plants, but this particular aspect of the problem was not included in the present study.

Summary

1. Oats were grown in gravel culture in a complete nutrient solution for a period of three weeks. The plants were then switched to solutions deficient in single nutrients. The plants were harvested three weeks later during the early stages of the jointing process.

2. Ascorbic acid was determined at the time of harvest. Riboflavin, calcium, magnesium, iron, phosphorus, potassium, and nitrogen were determined on oven-dry samples of tissue. Fresh weight and dry weight were also taken on the leaves, stems, and roots.

3. Mineral analysis showed that the deficient solutions had produced plants whose tissue was low in the nutrient omitted when compared with a control plant grown in a complete nutrient solution.

4. The leaves of the plants grown in the Mg- and K-deficient solutions contained a considerably higher concentration of ascorbic acid than the control plants. The stems of the Mg-deficient plants contained a higher concentration of ascorbic acid than the control plants. The evidence for stimulation of ascorbic acid synthesis in leaves low in potassium or magnesium was strengthened by the use of correlation analysis.

5. All mineral deficiency treatments used caused lower riboflavin concentration in the leaves and stems of the plants grown on them than did the complete nutrient solution. In the leaves, where the bulk of the riboflavin of the plant is located, the mineral deficiencies influenced riboflavin concentration to the greatest extent. The nitrate-deficient solution produced the lowest riboflavin concentration in leaf, stem, and roots. The influence of

nitrogen on riboflavin synthesis was also demonstrated by finding a high positive correlation between nitrogen and riboflavin concentrations in the leaves. The apparent influence of magnesium on riboflavin synthesis appears actually to be a nitrogen effect since leaves low in magnesium were also low in nitrogen.

6. Growth of leaves and stems in terms of dry substance was significantly correlated with the riboflavin concentration. Possible significance of this relationship is discussed.

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INTERRELATIONSHIPS OF CALCIUM, NITROGEN, AND PHOSPHORUS IN VEGETABLE CROPS[1]

S. H. WITTWER, R. A. SCHROEDER, AND WM. A. ALBRECHT

Coincident with the rapid advances being made in soil science, an increasing interest has been shown in the soil mechanisms as they control plant nutrition, growth, and crop production. One of the most influential factors involved is that of the interaction of nutrients. The equilibrium among ions in the soil and the culture solution has lately been designated as "nutrient-element balance." Emphasis has been placed on the interrelations of essential plant nutrients, on "antagonisms" between specific cations, and on the possible application of such relationships to fertilizer practices in the field.

In the nineteenth century WOLFF (37) noticed that with barley the greatest growth occurred in "complete" nutrient cultures. Excess potassium depressed yields. It was observed, however, that the depression in growth could be overcome by the addition of another nutrient. He noted also that sodium amendments offset the effects of excess potash. LAGATU and MAUME (16) recorded a decrease in the yield of grapes when potassium was omitted from an otherwise balanced fertilizer application. THOMAS (25, 26, 27) substantiated the work of LAGATU and MAUME, and his data further emphasized the importance of proper balance in fertilizer applications with reference to absorption.

Associated with nutrient balance are the frequently demonstrated cationic antagonisms. HOAGLAND (12), LUNDEGÅRDH (18), and RICHARDS (22) summarized the interactions existing in the absorption of potassium, calcium, magnesium, and sodium. Possible ways in which one element in nutrition may substitute for another are outlined by COOPER (6). A decrease in plant growth and an accentuation of mineral element deficiency symptoms by unbalanced soil cations have also been demonstrated by many investigators (7, 8, 17, 21, 28). The concept that a lack of balance may be more harmful to plant growth than a deficiency of two or more nutrients has been suggested. The reports of DAVIDSON and BLAKE (9), and of WAUGH, CULLINAN, and SCOTT (31), and recently those of BROWN (4) on nutrient balance in the peach bear this out. PHILLIPS, SMITH, and HEPLER (20) reached a similar conclusion with the tomato plant.

During the past six years at the Missouri Agricultural Experiment Station, the importance of nutrient balance in obtaining maximum response to fertilizer treatment has been repeatedly observed in nutritional studies with vegetables. This report deals with the yields of some vegetable crops as in-

[1] Missouri College of Agricultural Journal Series no. 1010.

244

fluenced by the balance of calcium, nitrogen, and phosphorus when the plants were grown in cultures of beidellite clay. This is a naturally occurring clay of which most of its readily available nutrient ions have been removed by leaching and H adsorption during continuous weathering.

Methods

Spinach, Swiss chard, lettuce, tampala, and tomato plants were grown under controlled greenhouse conditions in glazed gallon crocks using colloidal clay cultures (3) for nutrient media. Variable fertility levels were achieved by titrating the exchangeable ions, in the desired amounts and ratios, onto the original acid clay subsoil or B-horizon of Putnam silt loam, which has an exchange capacity of 28 milliequivalents per 100 grams, 12 of which are hydrogen. Of the remaining 16 milliequivalents of adsorbed nutrients, 12 are calcium and the remaining 4 are composed of smaller quantities of potassium and minor nutrient elements. Although these nutrient cations occupy a considerable portion of the total exchange capacity of the native subsoil, numerous biological tests have demonstrated its contribution of nitrogen and phosphorus to be nil, while practically none of the calcium and potassium is available for plant growth. Various nutrients, held on the clay in exchangeable form, may be provided for plants in any desired ratios and quantities simply by replacing the hydrogen on the clay with selected cations and by using the proper amount of prepared clay in the nutrient substrate. The pH values of the resulting media approximated 6.5. Stability of the clay and its naturally high content of replaceable hydrogen make its use, by simple additions of cations as exchanges for its hydrogen, very convenient for balanced nutrient studies.

The usual procedure followed in setting up the colloidal clay cultures was the preparation of a series of clay aliquots to which were added 5, 10, 20, and 40 milliequivalents of nitrogen in the form of ammonium nitrate. To each of these levels of nitrogen there was added calcium, as calcium acetate, in variable amounts to provide 0, 5, 10, 20, and 40 m.e. of calcium. This provided, then, twenty soil treatments giving four levels of nitrogen, each of which had combined with it five variable amounts of calcium as additions to the supply native in the initial clay. To each of these individual treatments were added other nutrients in constant quantities. The additions consisted of 20 m.e. each of potassium and phosphorus and 6 m.e. each of magnesium and sulphate (table I). Growth responses indicated that sufficient quantities of all trace elements are supplied by the native clay subsoil employed as an adsorptive media, the absolute amounts varying, of course, directly with the quantity of clay used. Effects of the variable quantities of colloid in the several treatments, since they might influence diversely the physical properties of the growing media, were reduced to a minimum by blending the clay with large quantities of pure sand or other chemically inert material. Single treatments were replicated at least three times, but usually ten times. Variations in plant growth within individual

treatments were extremely small, resulting from the degree of control exercised upon the chemical as well as the physical properties of the substrate.

In the more extensive studies involving variable levels of nitrogen, calcium, and phosphorus, aliquots of clay with ammonium nitrate and calicum acetate added in quantities to secure the desired ratios were prepared. Variable phosphorus levels were achieved without altering other nutrient levels, simply by adjusting the quantities of monobasic and dibasic potassium phosphates. Additional potassium, when needed, was supplied as the acetate. Control of nutrient levels was thus achieved with all nutrients other than nitrogen, calcium, and phosphorus constant for all treatments

TABLE I

NUTRIENTS ADDED TO CLAY TO PROVIDE VARIABLE LEVELS OF CALCIUM AND NITROGEN

TREATMENT	MILLIEQUIVALENTS PER PLANT						CLAY PER PLANT
	Ca	N	P	K	Mg	S	
	m.e.	m.e.	m.e.	m.e.	m.e.	m.e.	gm.
1	40	40	20	20	6	6	717
2	40	20	20	20	6	6	633
3	40	10	20	20	6	6	592
4	40	5	20	20	6	6	571
5	20	40	20	20	6	6	550
6	20	20	20	20	6	6	467
7	20	10	20	20	6	6	425
8	20	5	20	20	6	6	404
9	10	40	20	20	6	6	467
10	10	20	20	20	6	6	383
11	10	10	20	20	6	6	342
12	10	5	20	20	6	6	321
13	5	40	20	20	6	6	425
14	5	20	20	20	6	6	342
15	5	10	20	20	6	6	300
16	5	5	20	20	6	6	279
17	0	40	20	20	6	6	383
18	0	20	20	20	6	6	300
19	0	10	20	20	6	6	258
20	0	5	20	20	6	6	238

(table II). The amount of subsoil clay required to provide the exact exchange capacity for the added nutrients in each treatment was determined beforehand in terms of the known qualities of the clay. Putnam subsoil material was then mixed under moisture with the particular nutrients and homogeneously blended with either pure white quartz sand or "Zonolite."[2] Plants grown in the resulting mixtures were harvested and yields expressed in terms of fresh weights of the tops.

Results

The yields, expressed as fresh weights of spinach (Bloomsdale Long Standing), Swiss chard (Lucullus), and head lettuce (Iceberg), grown at variable levels of calcium and nitrogen with all other nutrient constant in

[2] A type of vermiculite widely used as an insulating material.

TABLE II

AMOUNTS OF NUTRIENT SALTS (M.E.) AND CLAY USED IN PROVIDING THREE LEVELS EACH OF NITROGEN, CALCIUM, AND PHOSPHORUS IN 27 POSSIBLE RATIOS

TREATMENT	VARIABLES			SALTS USED TO PROVIDE DESIRED MILLIEQUIVALENTS OF IONS												CLAY PER PLANT
	N	Ca	P	NH₄–NO₃		K(H₂)–PO₄		K₂(H)–PO₄		Ca–Ac		K–Ac		Mg–SO₄		
	m.e.	m.e.	m.e.	m.e.	m.e.	m.e.	m.e.	m.e.	m.e.	m.e.	m.e.	m.e.	m.e.	m.e.	m.e.	gm.
1	90	90	90	45	45	15	45	30	45	90	90			10	10	1583
2	90	90	45	45	45			30	45	90	90	15	15	10	10	1583
3	90	90	15	45	45			10	15	90	90	35	35	10	10	1583
4	90	45	90	45	45	15	45	30	45	45	45			10	10	1167
5	90	45	45	45	45			30	45	45	45	15	15	10	10	1167
6	90	45	15	45	45			10	15	45	45	35	35	10	10	1167
7	90	15	90	45	45	15	45	30	45	15	15			10	10	958
8	90	15	45	45	45			30	45	15	15	15	15	10	10	958
9	90	15	15	45	45			10	15	15	15	35	35	10	10	958
10	45	90	90	22½	22½	15	45	30	45	90	90			10	10	1396
11	45	90	45	22½	22½			30	45	90	90	15	15	10	10	1396
12	45	90	15	22½	22½			10	15	90	90	35	35	10	10	1396
13	45	45	90	22½	22½	15	45	30	45	45	45			10	10	1021
14	45	45	45	22½	22½			30	45	45	45	15	15	10	10	1021
15	45	45	15	22½	22½			10	15	45	45	35	35	10	10	1021
16	45	15	90	22½	22½	15	45	30	45	15	15			10	10	771
17	45	15	45	22½	22½			30	45	15	15	15	15	10	10	771
18	45	15	15	22½	22½			10	15	15	15	35	35	10	10	771
19	15	90	90	7½	7½	15	45	30	45	90	90			10	10	1271
20	15	90	45	7½	7½			30	45	90	90	15	15	10	10	1271
21	15	90	15	7½	7½			10	15	90	90	35	35	10	10	1271
22	15	45	90	7½	7½	15	45	30	45	45	45			10	10	896
23	15	45	45	7½	7½			30	45	45	45	15	15	10	10	896
24	15	45	15	7½	7½			10	15	45	45	35	35	10	10	896
25	15	15	90	7½	7½	15	45	30	45	15	15			10	10	646
26	15	15	45	7½	7½			30	45	15	15	15	15	10	10	646
27	15	15	15	7½	7½			10	15	15	15	35	35	10	10	646

all treatments, are given (table III). It is noteworthy that the lowest production occurred in spinach and chard when the highest level of calcium (40 m.e.) was combined with the lowest nitrogen level (5 m.e.) in treatment 4. This reduction in growth was much greater than with a deficiency of both elements, as in treatment 16 or 20. With the highest amount of calcium (40 m.e.) the successive increases of applied nitrogen gave a general increase in the yield with all crops. At the reduced calcium levels of 10 and 20 m.e. and especially at the 0 and 5 m.e. levels increases in the nitrogen from 5 to 10 m.e. and from 10 to 20 m.e. also gave a significant rise in yield, but a further increase to 40 m.e. of nitrogen cut production rather sharply

TABLE III

YIELDS OF SPINACH, SWISS CHARD, AND LETTUCE ACCORDING TO VARIABLE
LEVELS OF CALCIUM AND NITROGEN

TREATMENT	VARIABLES		FRESH WEIGHTS*		
	Ca	N	SPINACH	SWISS CHARD	LETTUCE
	m.e.	m.e.	gm.	gm.	gm.
1	40	40	234.4	634.6	580.0
2	40	20	170.8	487.3	393.0
3	40	10	85.0	391.6	171.0
4	40	5	36.5	32.0	239.0
5	20	40	320.5	486.4	431.0
6	20	20	220.1	693.5	489.0
7	20	10	108.5	333.8	240.0
8	20	5	65.4	212.7	155.5
9	10	40	179.1	233.3	529.0
10	10	20	229.0	243.4	485.0
11	10	10	138.5	215.2	220.0
12	10	5	114.9	171.5	161.0
13	5	40	128.6	179.8	158.0
14	5	20	205.1	133.8	400.0
15	5	10	153.1	116.6	258.0
16	5	5	60.1	104.6	181.0
17	0	40	67.8		67.0
18	0	20	197.3		260.0
19	0	10	99.9		155.0
20	0	5	76.7		60.0

* Grams per ten plants.

in all three crops. Yields were a direct function of the total nutrient supply only when the variables were properly balanced. They point out that a high calcium level must be accompanied by adequate amounts of nitrogen, and a high nitrogen level by sufficient amounts of calcium. Results obtained by growing spinach and Swiss chard at levels of 5, 15, and 45 m.e. each of nitrogen, calcium, and phosphorus in all 27 combinations are summarized (table IV). Potassium was held constant at 20 m.e. and magnesium and sulphate at 5 m.e. for each treatment.

Although responses to nitrogen and phosphorus were outstanding, calcium exerted appreciable influence. Considering calcium, the largest growth

occurred at the moderate (15 m.e.) level of calcium combined with the highest of nitrogen (treatments 4–6 inclusive). At a low nitrogen level for both crops (treatments 19–27) and at a medium level of nitrogen for Swiss chard (treatments 10–18) an improvement in growth was obtained when the calcium was reduced to its lowest figure. A rather marked reduction in the yield of both vegetables was noted as the calcium was increased in combination with the lowest nitrogen level.

TABLE IV

YIELDS OF SPINACH AND SWISS CHARD ACCORDING TO VARIABLE LEVELS OF
NITROGEN, CALCIUM, AND PHOSPHORUS

TREATMENT	VARIABLES			FRESH WEIGHT*	
	N	Ca	P	SPINACH	SWISS CHARD
	m.e.	*m.e.*	*m.e.*	*gm.*	*gm.*
1	45	45	45	168.8	227.7
2	45	45	15	197.6	202.6
3	45	45	5	45.5	93.5
4	45	15	45	213.7	232.0
5	45	15	15	176.0	181.0
6	45	15	5	53.8	114.3
7	45	5	45	145.5	206.3
8	45	5	15	171.5	178.7
9	45	5	5	68.3	88.2
10	15	45	45	101.5	67.8
11	15	45	15	82.7	53.4
12	15	45	5	70.1	41.5
13	15	15	45	129.6	68.2
14	15	15	15	102.2	69.1
15	15	15	5	87.8	53.0
16	15	5	45	104.7	112.0
17	15	5	15	103.1	92.3
18	15	5	5	78.5	65.3
19	5	45	45	28.2	20.1
20	5	45	15	18.7	12.8
21	5	45	5	14.7	9.6
22	5	15	45	43.5	24.5
23	5	15	15	24.4	18.0
24	5	15	5	19.5	20.0
25	5	5	45	35.0	35.2
26	5	5	15	28.6	21.6
27	5	5	5	25.4	15.2

* Grams per five plants.

Increments of phosphorus applied to cultures low in nitrogen (5 and 15 m.e.) failed to give the yield increases possible at high nitrogen (45 m.e.). Conversely, the response to increasing nitrogen was largely governed by the phosphorus supply. When additional calcium was supplied to cultures low in phosphorus, the deficiency of phosphorus was accentuated (compare treatments 12, 15, 18 and 21, 24, 27). The best nutrient-element balance for yield increase was attained in treatment 4, with 45 m.e. as the level of nitrogen and phosphorus and with 15 m.e. as that for calcium. The lack
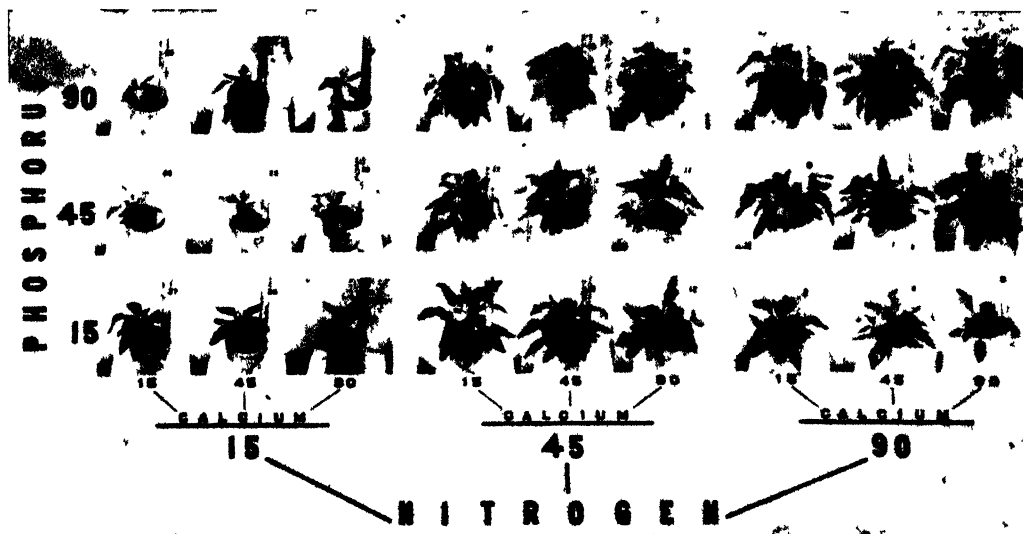
Fig. 1. Tomato plants at variable levels of nitrogen, calcium, and phosphorus. (Numbers indicate m.e. of respective nutrients applied per plant.)

of balance with reference to plant growth was most evident in treatment 21 where 45 m.e. of calcium were combined with 5 m.e. each of nitrogen and phosphorus.

Responses by the tomato plant (variety Marglobe) to combinations of three variable levels of nitrogen, calcium, and phosphorus are portrayed (fig. 1). Yields expressed as fresh weights of vegetation are given (table V). The plants were grown at 5, 15, and 45 m.e. each of nitrogen, calcium, and phophorus in 27 different combinations. It is evident that a lack of balance among the nutrients was more detrimental than a deficiency in all.

TABLE V

YIELDS OF TOMATO PLANTS ACCORDING TO VARIABLE LEVELS OF NITROGEN, CALCIUM, AND PHOSPHORUS*

PHOS-PHORUS	NITROGEN 5 M.E.			NITROGEN 15 M.E.			NITROGEN 45 M.E.		
	CALCIUM, M.E.			CALCIUM, M.E.			CALCIUM, M.E.		
	5	15	45	5	15	45	5	15	45
m.e.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
45	68.6	76.8	57.6	166.0	153.6	169.4	442.0	567.6	516.2
15	60.4	61.0	158.0	202.8	214.8	200.4	345.2	477.6	377.4
5	60.8	48.8	55.4	160.4	119.6	41.2	29.0	25.4	21.2

* Grams per three plants.

The data indicate that phosphorus deficiency in the plants at the 5 m.e. level of this nutrient was greatly accentuated by high calcium and high nitrogen. With the low level of phosphorus, an increase in the nitrogen from 15 to 45 m.e. depressed the yields.   Similarly, at 5 m.e. of phosphorus (and constant osmotic pressure) the increases of calcium gave decreasing yields.

The phosphorus response in tomato nutrition (fig. 1), was dependent almost entirely upon the soil nitrogen supply.   By contrast, addition of nitrogen to the soil decreased or increased yields depending on the amount of phosphorus present.   With respect to increasing amounts of calcium applied at a high nitrogen and a high or medium phosphorus level, the effects were first an increase and then, with further additions of calcium, a decrease in production.   At low phosphorus and high nitrogen levels, the calcium additions progressively diminished yields.   In the tomato as with other

Fig. 2.   Tampala plants at variable levels of nitrogen, calcium, and phosphorus. (Numbers indicate m.e. of respective nutrients applied per plant.)

crops, growth was determined not so much by the total quantities of nutrients added as by the balance relations existing among the elements.

Tampala (*Amaranthus gangeticus*) was grown at nutrient levels of 15, 45, and 90 m.e., each of nitrogen, calcium, and phosphorus in all possible combinations.   The nutrient salt additions and clay aliquots were set up in accordance with the scheme as outlined (table II).   The resulting plant growth and yields of the crop are presented (fig. 2 and table VI).   The influence on the yields by a proper balance among the nutrients was again clearly demonstrated.   Phosphorus additions either increased, had little effect on, or decreased the growth, depending on the nitrogen and calcium levels.   At 15 m.e. of nitrogen, increased phosphorus depressed the yields at all three calcium levels; at 45 m.e. of nitrogen there was little influence, whereas at 90 m.e. of nitrogen, a very significant yield increase was noted. When only 15 m.e. of nitrogen were provided, the addition of calcium accen-

TABLE VI

YIELDS OF TAMPALA PLANTS ACCORDING TO VARIABLE LEVELS OF NITROGEN, CALCIUM, AND PHOSPHORUS*

| PHOS-PHORUS | NITROGEN 15 M.E. | | | NITROGEN 45 M.E. | | | NITROGEN 90 M.E. | | |
| | CALCIUM, M.E. | | | CALCIUM, M.E. | | | CALCIUM, M.E. | | |
	15	45	90	15	45	90	15	45	90
m.e.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
90	55.1	52.9	42.1	227.0	230.3	219.9	332.5	372.3	362.9
45	63.8	57.1	52.6	194.7	273.4	265.2	316.7	319.8	333.2
15	87.7	69.5	62.1	184.6	206.9	169.5	186.8	161.7	90.4

* Grams per five plants.

tuated nitrogen deficiencies at all three phosphorus levels. Plants grown with 90 m.e. of nitrogen and 15 m.e. of phosphorus gave noticeably reduced yields, especially when this lack of balance was aggravated by calcium additions.

Discussion

In the experiments on nutrient-element balance for growing plants by the sand and/or solution culture method reported to date, it was impossible to control total concentrations of solutions (4) and hence their osmotic pressures as the ions were varied. On the other hand, if the concentrations were held constant (28), then an increase or decrease in one nutrient ion required that other ions be varied (11, 24). Exact interpretation of results in terms of a single ion is extremely difficult with sand and solution cultures.

As an approach to the accurate study of nutrient balance, the clay technique of plant growing is admirably adapted. The advantages of this method, and of using other ion adsorptive materials, for refined control of nutritional experiments with vegetables have previously been outlined (1, 2, 3, 5, 14). Not only do ion adsorptive materials permit the addition of each major cation separately as a carbonate, hydroxide, or acetate without changing other ionic concentrations, but clay holds also the anion of phosphorus in an adsorbed form (13). Studies conducted by GRAHAM and ALBRECHT (10) have shown that the nitrate anion may be adsorbed by certain synthetic resins. The adsorbed nitrate was available to plants as readily as were adsorbed cations. Corn plants grew equally well when nitrogen was added in the adsorbed form as in solution. JENNY (15) demonstrated by growing lettuce that adsorbed nitrates were superior to soluble nitrates in amounts above 20 m.e. per plant. The possibility of combining cation and anion adsorptive materials should not be overlooked in plant nutritional studies.

Physical structures of the clay cultures have not gone without consideration. Variations in nutrient levels and colloid content must have a minimum effect on the physical properties of the soil. When combined with

large quantities of leached white sand, the clay adheres to the surface of sand particles making up only a small fraction of the total body. Thus the physical properties of the media are not greatly affected. Even a more ideal culture mixture with reference to physical structure can be had by blending clay with "Zonolite." This heat-treated mineral silicate is readily available in several forms as a common insulating material, has a high water-holding capacity, and is practically inert chemically. Its use in combination with clay in nutrient cultures of restricted volume facilitates the addition of large quantities of clay loaded with nutrient ions. At the same time, a physical soil structure is created which is easily penetrable throughout by plant roots, has a high water-holding capacity, and is sufficiently porous for adequate aeration.

Nutrient balance only as it affects yields of some vegetable crops has been emphasized in this report. A few variable levels and combinations of levels of nitrogen, calcium, and phosphorus have thus far been utilized in the approach to fertility balance in nutrition of vegetable crops. These are the nutrient elements most commonly deficient in Missouri soils. An influence of the balance of nitrogen and calcium on the vitamin, mineral, and oxalate contents has been shown (**32, 33, 34**). Some of the possibilities of disease (**19, 23, 29, 30, 36**) and insect (**35**) control through balanced soil fertility have been demonstrated. The approach offered by the colloidal clay technique of growing plants, provides a means of studying nutrient balance relations in their many ramifications, heretofore not fully appreciated by students of plant nutrition.

Summary

1. The influence of nutrient-element balance on the growth and production of vegetation in spinach, Swiss chard, lettuce, tampala, and tomato was studied by means of growing the plants in cultures prepared by blending colloidal clay with sand or chemically inert "Zonolite." Variable levels of nitrogen, calcium, and phosphorus were supplied.

2. Growth responses by the plants were found to be dependent on relative proportion as well as absolute amounts of variable nutrient elements present in the substrate. Yields were increased, not affected, or depressed by a particular ion, depending on the levels at which the other ions were present in the media. A lack of balance was demonstrated to be more detrimental to plant growth than a deficiency of all the variable nutrients.

3. Advantages of colloidal clay cultures as a means of approach to the study of nutrient-element balance are outlined. Maximum flexibility of variables is possible without concomitant alterations in osmotic pressures or physical properties of the media.

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ETHER–SOLUBLE ORGANIC ACIDS OF MATURE VALENCIA ORANGE LEAVES[1]

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(WITH TWO FIGURES)

Introduction

In a series of studies (**16, 18, 20**) on the organic acids of citrus fruits, the authors have pointed out that large amounts of organic acids exist in the juice of the pulp, as compared with exceptionally low concentrations in the peel. The vascular system of the fruit is confined chiefly to the mesocarp (albedo), and the latter thus serves the important function of transporting water and solutes from the tree to the juice vesicles of the pulp. Since these vesicles contain relatively large amounts of organic acids, it would appear that higher concentrations than are present should occur in the mesocarp, that is, if the total organic acid radical is transferred from leaves to vesicles. This situation led to the postulation that the organic acids of the juice are possibly synthesized in the pulp vesicles rather than in the leaves and subsequently transferred to the pulp. Quantitative determination and identification of the organic acids present in the leaves should provide fundamental data necessary for a solution to this problem.

The present investigation has been concerned with the extraction of the organic acids from ground, dried Valencia orange leaves with absolute ethyl ether, and with the subsequent determination of total and individual organic acids in the water solution of the ether extract. In addition, water-soluble organic acids were extracted directly from aliquot portions of the dried leaf samples, and these data were compared with the total and individual acids extracted with ether. This information revealed the amounts of organic acids present in the leaves in soluble and insoluble states. The influence of these factors on the buffer system of the leaves is discussed. Furthermore, certain relationships are proposed concerning the synthesis and translocation of organic acids from the leaves to the fruit.

Materials and methods

PREPARATION OF SAMPLES

For these experiments, 300- to 500-gram samples of mature Valencia orange leaves (1 to 2 years old) were taken from 20-year-old trees in plots located at the University of California Citrus Experiment Station. Leaf samples were purposely picked at stated hours (9:00 A.M. and 1:30 P.M.), over a period of five days, beginning at 1:30 P.M. June 10 and ending at 9:00 A.M. June 14.

The freshly picked leaves were wiped free of dirt with a soft cotton cloth, placed in wire baskets, and dried in a ventilated oven at 65° C. When thoroughly dried, the leaf samples were ground to a finely divided state in a Wiley mill. The total nonvolatile organic acids were extracted from these dried, ground samples with absolute ethyl ether.

Water extracts on which the soluble organic acids were determined were obtained by placing 2 gm. of dried leaves and 125 ml. of water in a round-bottom flask and allowing extraction, with frequent shaking, on a water bath for 4 hours. The extract was filtered from the residue, and the filtrate and washings were diluted to a 200-ml. volume. Aliquot portions of this extract were used for determining the soluble citric, malic, and oxalic acids, and the soluble potassium and calcium. The pH values of the water extract were also determined on aliquot portions of this solution.

Leaf sap was obtained by cutting the fresh leaves into small pieces, placing them in glass jars with covers, and freezing the tissue for 48 to 60 hours at $-25°$ C. After thawing, the sap was extracted from the leaves by means of a hydraulic press, at a pressure of 20,000 pounds per square inch. Sap was centrifuged, and the supernatent liquid was used for determining the titration curve.

CHEMICAL METHODS

Organic acids were isolated from the dried leaf samples by the method of PUCHER, VICKERY, and WAKEMAN (10). A known weight of ground, dried leaves was acidified to pH 1 with sulphuric acid (4 N) and mixed thoroughly with asbestos. The material was subsequently extracted with absolute ether (H_2O_2 free) for 30 hours. Water was added to the extract and the ether was carefully evaporated from the solution, leaving the organic acids in the aqueous phase. The water solution containing the organic acids was filtered into a volumetric flask and diluted to final volume. Aliquot portions of this solution were used for analysis.

Total organic acids were determined by titrating the water solutions of the ether extracts between the limits of pH 7.8 and pH 2.6, according to the method of VAN SLYKE and PALMER (21). Since oxalic acid reacted as a monobasic acid under these conditions, a correction was made by determining, independently, the amount of oxalic acid in the samples. Suitable aliquots, depending upon the oxalic acid content, were measured into 100-ml. beakers and acidified to Congo red with 0.5 N HCl. The material that flocculated on standing was filtered off on a fine-sintered crucible and washed with water. The precipitation and titration of the oxalate from this clear liquid were carried out according to the procedure of PUCHER, VICKERY, and WAKEMAN (10).

Titration values of the sap and water extracts of the dried leaf samples were determined at 23° C. with a Beckman glass-electrode pH meter.

The quantitative determination of citric acid, free and combined, was made on the samples by the pentabromacetone method of PUCHER et al. (11). The sample was heated with H_2SO_4 to convert the combined citrates to free

citric acid, and the citric acid was oxidized to pentabromacetone by $KMnO_4$ in the presence of KBr. After extraction of the pentabromacetone with petroleum ether, the bromide ion was liberated with Na_2S and subsequently titrated with standard $AgNO_3$. Citric acid in the original sample was calculated from the titration.

Malic acid was determined on aliquot portions of this solution by the pentabromacetone method of PUCHER et al. (11). The principle of the method involved oxidation of malic acid with $KMnO_4$ in the presence of KBr, to a bromine compound volatile with steam. This compound reacts with 2,4-dinitrophenylhydrazine to give a water-insoluble product which is soluble in pyridine. A pyridine solution of this substance, when correctly diluted with water and made alkaline with NaOH, develops a blue color proportional to the amount of malic acid present.

Organic acids of the water extract were precipitated from an alcohol solution (85%) of the extract with lead acetate, according to the procedure of HARTMANN and HILLIG (3). The precipitate was washed with alcohol, suspended in water, and freed of lead by passing H_2S through the solution. The lead sulphide was filtered off and washed with water. The filtrate and washings were combined and diluted to a known volume. Aliquot portions of this solution were used for determining the organic acids that were precipitated with lead acetate.

The total and water-soluble calcium and potassium were determined on the dried leaf samples. Calcium was determined volumetrically by treating the oxalate with dilute H_2SO_4 and subsequently titrating the liberated oxalic acid with standard potassium permanganate. Potassium was determined gravimetrically according to the method described by WILCOX (22).

Results

ETHER-SOLUBLE ORGANIC ACIDS OF THE LEAVES

The amounts of the various organic acids obtained from dried Valencia orange leaves by continuous extraction with absolute ethyl ether are shown (table I). As the extraction was performed at a pH of 1, the ether extract includes, in the free form, the total organic acid radical. The various acid constituents in these mature leaves did not vary significantly with respect to the time of day in which the samples were collected.

Total acids extracted from the leaves with ether were determined by titrating the aqueous solutions of the ether extracts between pH 7.8 and pH 2.6. Concentration of total acids in the leaf samples ranged from 1.895 m.e. to 2.492 m.e. per gram of dry matter. The distinctive feature of table I is that the concentrations of malic and oxalic acids are shown to be more than twice that of citric acid. The sum of the citric, malic, and oxalic acids is not equal to the total acids extracted from the leaves with ether. Undetermined acids in the samples varied from 10.06% to 24.08% of the total acids present. Apparently one (or more) unknown organic acid is present in this undetermined fraction. It should be emphasized at this point that

TABLE I

Ether-soluble organic acids of mature Valencia orange leaves

Sample No.	Moisture, Fresh Weight Basis	Total Acids Titrated (pH 7.8 to 2.6)	Citric Acid			Malic Acid			Oxalic Acid			Sum of Citric, Malic, and Oxalic Acids		Undetermined Acids	
	%	m.e./gm.*	mg./gm.†	m.e./gm.*	%‡	mg./gm.†	m.e./gm.*	%‡	mg./gm.†	m.e./gm.*	%‡	m.e./gm.*	%‡	m.e./gm.*	%‡
1	56.74	1.895	15.24	0.238	12.56	41.49	0.619	32.66	31.46	0.699	36.89	1.556	82.11	0.339	17.89
2	56.22	2.028	18.57	0.290	14.30	40.02	0.597	29.44	42.17	0.937	46.20	1.824	89.94	0.204	10.06
3	56.29	2.328	19.72	0.308	13.23	45.51	0.679	29.17	41.81	0.929	39.91	1.916	82.30	0.412	17.70
4	61.10	2.492	18.57	0.290	11.64	55.03	0.821	32.95	37.94	0.843	33.83	1.954	78.41	0.538	21.59
5	61.52	2.259	15.24	0.238	10.54	36.73	0.548	24.26	41.81	0.929	41.12	1.715	75.92	0.544	24.08
6	59.36	2.303	13.90	0.217	9.42	46.72	0.697	30.26	41.63	0.925	40.17	1.839	79.85	0.464	20.15
7	60.38	2.253	19.72	0.308	13.67	47.99	0.716	31.78	37.22	0.827	36.71	1.851	82.16	0.402	17.84
8	59.88	2.247	18.57	0.290	12.91	51.28	0.765	34.05	40.68	0.904	40.23	1.959	87.18	0.288	12.82
Mean	58.94	2.226	17.44	0.272	12.28	45.60	0.680	30.57	39.34	0.874	39.38	1.827	82.23	0.399	17.76

* Milliequivalents per gram of dry matter,
† Milligrams per gram of dry matter.
‡ Percentage of total acids.

the total acids extracted from the leaves by ether were corrected for the phosphate and H_2SO_4 occurring in the extract.

Although the different acid fractions showed considerable variation between samples, as a whole the results showed definite uniformity. Since these leaves were fully mature, their respiration rates and metabolic reactions were probably much lower than similar reactions in young leaves. If these mature leaves had had high respiration rates, it is probable that large differences in the concentration of organic acids would have occurred between the different samples.

WATER-SOLUBLE ORGANIC ACIDS OF THE LEAVES

The water-soluble organic acids in the dried leaf samples are shown (table II). Obviously, these determinations were made on the same leaf
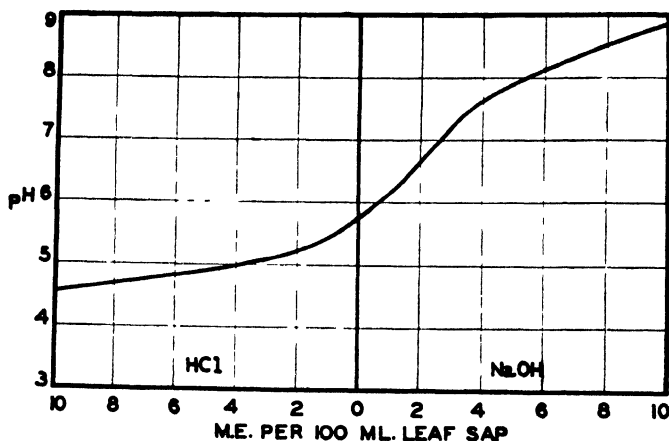
FIG. 1.   Titration curve of undiluted sap of mature Valencia orange leaves.

samples used in obtaining the data for table I. Total soluble acids in the water extract varied from 1.056 m.e. to 1.478 m.e. per gram of dry matter. Active acidity of the water extracts varied between pH 5.35 and pH 5.55. These values are slightly lower (0.3 to 0.4 of a pH) than those recorded for undiluted leaf sap (fig. 1). No presumption is made that the pH values of either the water extracts or expressed saps represent the pH of the uninjured cells. Under such experimental conditions as these, all pH and concentration gradients within and between the cells of the leaves are eliminated. The ionic concentrations of the water extracts and of the expressed saps are quite different from the total amount soluble in the sap of uninjured cells. The pH values of these systems therefore represent determinations made on a composite sample of the soluble constituents that affect the acidity and buffer properties of the leaf tissue.

The amounts of water-soluble citric acid in the different samples varied from 13.90 mg. to 19.72 mg. per gram of dry matter. In all samples except one (no. 5), more than 90% of the total citric acid was water soluble. Similar values for malic acid reveal the high solubility of this acid in the water extract. For some unknown reason, however, the values for malic acid were slightly more variable than those for citric acid. It is possible

TABLE II

WATER-SOLUBLE ORGANIC ACIDS OF MATURE VALENCIA ORANGE LEAVES

SAMPLE NO.	pH OF WATER EXTRACTS	TOTAL SOLUBLE ACIDS TITRATED m.e./gm.*	CITRIC ACID mg./gm.†	CITRIC ACID m.e./gm.*	CITRIC ACID %‡	MALIC ACID mg./gm.†	MALIC ACID m.e./gm.*	MALIC ACID %‡	OXALIC ACID mg./gm.†	OXALIC ACID m.e./gm.*	OXALIC ACID %‡	SUM OF CITRIC, MALIC, AND OXALIC ACIDS m.e./gm.*	SUM OF CITRIC, MALIC, AND OXALIC ACIDS %§
1	5.40	1.056	13.90	0.217	91.18	39.01	0.582	94.02	1.85	0.041	5.87	0.840	79.55
2	5.35	1.088	18.57	0.290	100.00	38.48	0.574	96.15	1.62	0.036	3.84	0.900	82.72
3	5.48	1.379	19.72	0.308	100.00	40.02	0.597	87.92	1.85	0.041	4.41	0.946	68.60
4	5.52	1.478	18.57	0.290	100.00	37.47	0.559	68.09	1.62	0.036	4.27	0.885	59.88
5	5.50	1.182	13.00	0.203	85.29	36.00	0.537	97.99	1.40	0.031	3.34	0.771	65.23
6	5.55	1.232	13.45	0.210	96.77	37.47	0.559	80.20	1.31	0.029	3.14	0.798	64.77
7	5.55	1.182	18.57	0.290	94.16	36.00	0.537	75.00	1.40	0.031	3.75	0.858	72.59
8	5.50	1.232	17.48	0.273	94.14	37.00	0.552	72.16	1.31	0.029	3.21	0.854	69.32
Mean	5.48	1.229	16.66	0.260	95.19	37.68	0.562	83.94	1.55	0.034	3.98	0.857	70.33

* Milliequivalents per gram of dry matter.
† Milligrams per gram of dry matter.
‡ Soluble acid as percentage of total citric, malic, and oxalic acids, respectively (see table I).
§ Percentage of total soluble acids.

that these variations may be attributed to the highly active character of malic acid biochemically; its concentration in the leaves is influenced by diurnal changes as well as by methods of treatment previous to analysis.

The high solubility of citric and malic acids in the water extracts indicates that these two acids are also soluble in the leaf cells. That citric and malic acids are soluble in the extract in the presence of calcium ions is shown by the data in table III. In acid medium calcium ions do not precipitate citric and malic acids. In citrus leaf sap, which has an approximate pH of 5.8, citric and malic acids are soluble in the presence of calcium and free to migrate and participate in the metabolic processes of the cells.

In comparison with the total oxalic acid in the leaves (table I), the amount soluble in water, in the presence of soluble calcium, was relatively small (1.31 mg. to 1.85 mg. per gram of dry matter). This represents the approximate limit of solubility under these experimental conditions. Results show that most of the oxalic acid in mature Valencia orange leaves is in the cells as insoluble calcium oxalate. Some of the insoluble oxalate may be in the form of magnesium salt. This view is deducted from the results of experiments of PIERCE and APPLEMAN (8), who found that magnesium oxalate forms a supersaturated solution.

Water-soluble citric, malic, and oxalic acids are not equal in concentration to total acids in the water extract. The undetermined acids in the water extract are composed of some inorganic acid salts, and perhaps of other organic acid radicals.

RELATION OF SOLUBLE AND INSOLUBLE CALCIUM AND POTASSIUM TO THE OXALIC AND TOTAL ACIDS OF THE LEAVES

In general, the excess inorganic cations (over anions) of leaf tissue are highly correlated with the ether-soluble organic acids (8, 13), and the insoluble oxalates are highly correlated with the insoluble calcium (5, 6). In the present experiments no attempt was made to determine the cation-anion balance, but the data recorded in table III reveal certain pertinent facts about the potassium and calcium in relation to the acid constituents and their buffer properties in the leaves. The sum of calcium and potassium accounts for more than 95% of the total inorganic cations in these samples. The remainder of the inorganic cations is composed chiefly of magnesium and sodium.

Total calcium in the different samples varied from 1.882 m.e. to 2.017 m.e. per gram of dry matter. The calcium content of citrus leaves increases with the growth and age of the tissue (7); therefore, if these leaves had been older (for example, 3 years of age) the calcium concentration would have been much higher. Water-soluble calcium amounted to an average of 44.76% of the total calcium. More than half of the total calcium is combined in the leaves in an insoluble form. Again, it is essential to draw attention to the low concentration of oxalic acid in the water extracts (3.98% of total oxalic acid). Consequently, nearly all the oxalic acid in the leaves is in the form of insoluble oxalates. This condition in the leaves confirms the

TABLE III

THE TOTAL AND WATER-SOLUBLE CALCIUM, POTASSIUM, OXALIC, AND TOTAL ACIDS OF MATURE VALENCIA ORANGE LEAVES

SAMPLE NO.	TOTAL CONSTITUENTS						WATER-SOLUBLE CONSTITUENTS								WATER-SOLUBLE TOTAL ACIDS AND TOTAL OXALIC ACID
	Ca	K	OXALIC ACID		TOTAL ACIDS		Ca		K		OXALIC ACID		TOTAL ACIDS		
	m.e./gm.*	m.e./gm.*	m.e./gm.*	%†	m.e./gm.*		m.e./gm.*	%‡	m.e./gm.*	%‡	m.e./gm.*	%‡	m.e./gm.*	%†	%
1	1.882	0.186	0.699	36.89	1.895		0.845	44.90	0.182	97.85	0.041	5.87	1.056	55.73	92.62
2	1.916	0.175	0.937	46.20	2.028		0.902	47.08	0.171	97.71	0.036	3.84	1.088	53.65	99.85
3	1.938	0.164	0.929	39.91	2.328		0.898	46.34	0.162	98.78	0.041	4.41	1.379	59.24	99.15
4	2.017	0.148	0.843	33.83	2.492		0.919	45.56	0.145	97.97	0.036	4.27	1.478	59.31	93.14
5	1.907	0.186	0.929	41.12	2.259		0.811	42.53	0.185	99.46	0.031	3.34	1.182	52.32	93.44
6	1.897	0.166	0.925	40.17	2.303		0.870	45.86	0.165	99.40	0.029	3.14	1.232	53.50	93.67
7	1.907	0.163	0.827	36.71	2.253		0.827	43.37	0.157	96.32	0.031	3.75	1.182	52.46	89.17
8	1.917	0.170	0.904	40.23	2.247		0.813	42.41	0.164	96.47	0.029	3.21	1.232	54.83	95.06
Mean	1.923	0.170	0.874	39.38	2.226		0.861	44.76	0.166	98.00	0.034	3.98	1.229	55.13	94.51

* Milliequivalents per gram of dry matter.
† Percentage of total acids.
‡ Percentage of the respective total constituent in the leaves.

generalization that insoluble oxalates are correlated with insoluble calcium. If the sum of the water-soluble calcium and that combined with the oxalic acid be subtracted from total calcium, there remains a portion of insoluble calcium to combine with other substances (calcium pectate, *etc.*) of the leaves. The increase in insoluble oxalates with the growth and age of leaves is similar to the increase in total calcium. Calcium used in forming insoluble oxalates is not available for metabolic purposes. Apparently, the pH within the cells of citrus leaves never becomes sufficiently low to put calcium oxalate into solution. If this form of calcium is to be utilized by the plant, an enzymatic mechanism or some other process would have to change the insoluble calcium oxalates to other soluble compounds.

Nearly all the potassium in these leaf samples is water soluble (96.32% to 99.46%). This is evidence that the potassium salts are in the ionic form. Potassium salts of the organic acids are soluble in water. As these salts are soluble in the leaves, they contribute a considerable part of the buffer capacity of the leaf sap. The type of buffer curve exhibited by the sap is shown (fig. 1). Concentration of free acids, organic and inorganic, in the sap is relatively small and is represented by the portion of the curve between zero and the milliequivalents of NaOH required to bring the system to a pH of 8.20. The acid titration curve is recorded only to a pH of 4.60, and to 10 m.e. HCl per 100 ml. of leaf sap. From this point to a pH of 2.0, the curve slopes very gradually and is not of sufficient importance for consideration in these studies.

Water-soluble acids in the leaf samples average 55.13% of the total ether-soluble organic acids; the amount of organic acids insoluble in water is therefore 44.87%. Note has already been made of the high concentration of oxalic acid in the leaves, most of which is insoluble in water. The sum of the water-soluble acids and the total oxalic acid is nearly equal to the total acids of the leaves. In other words, oxalic acid accounts for nearly all the insoluble organic acids in the leaves.

ACID CONSTITUENTS OF THE LEAVES COMPARED WITH THOSE
OF THE FRUIT JUICE AND PEEL

An interesting comparison is shown (fig. 2), in that the total, citric, malic, and oxalic acids are given for mature leaves and for the juice and peel of mature Valencia orange fruits. Leaves contained the highest concentrations of total, malic, and oxalic acids; juice, however, contained the highest concentration of citric acid. The peel contained the lowest concentrations of total and citric acids; the juice contained the lowest concentrations of malic and oxalic acids. Special attention should be drawn to the exceptionally low concentration of citric acid in the peel. As already shown, the sum of the citric, malic, and oxalic acids is not equal to the total acids of the leaves, the undetermined fraction amounting to approximately 15 m.e. per gram of fresh weight. The determined fraction is composed chiefly of malic and oxalic acids, accompanied by a small amount of citric acid. As

in the leaves of most plants, the concentration of malic acid exceeds that of citric acid.

The exceptionally low concentration of organic acids in the peel of citrus fruits, in comparison with that in the leaves and juice, is highly suggestive of the location of organic acid synthesis in citrus fruits. These fruits, unlike pomaceous fruits, have no fleshy centers permeated by fibrovascular bundles. The vascular system is chiefly confined to the mesocarp (spongy parenchyma of the peel), and therefore serves the important function of transporting water and solutes from the tree to the pulp vesicles. These conclusions have
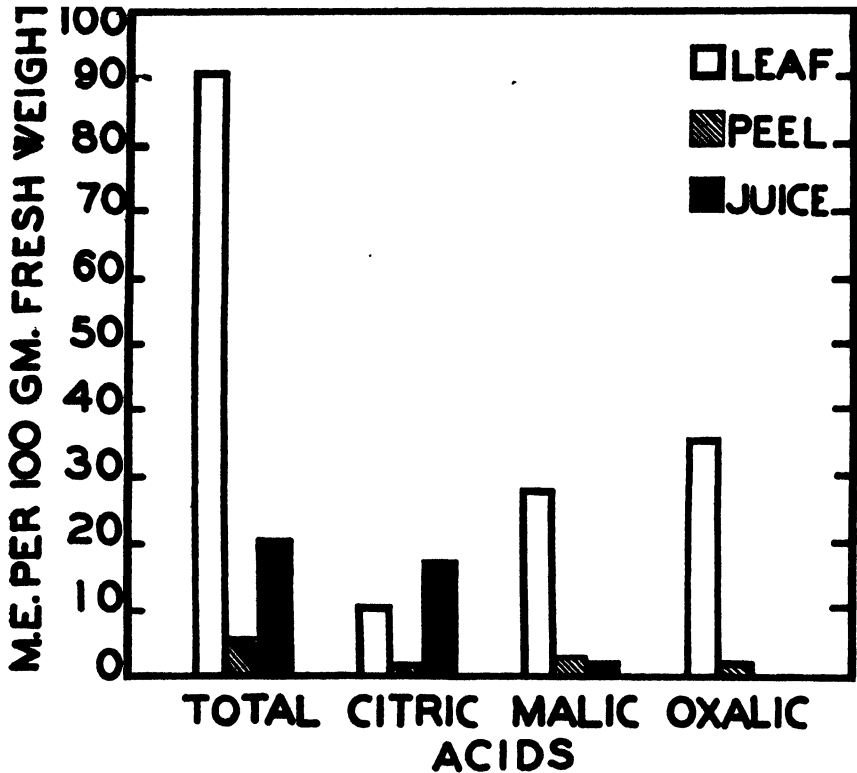
FIG. 2. Comparison of organic acids in mature leaves and in peel and juice of mature fruits of the Valencia orange.

been substantiated by the experiments of HODGSON (4), of BARTHOLOMEW (1), and of BARTHOLOMEW and REED (2), who showed the existence of an equilibrium between the water in citrus trees and in their fruits. This equilibrium is manifested by the diurnal changes in volume of fruits on trees subjected to an environment conducive to excessive leaf evaporation. Results of these studies led REED (14) to conclude that the translocation of liquids in citrus fruits is through the layers of hydrophilic colloids on the walls of the mesocarp, and that the juice vesicles in the pulp of the fruit exert sufficient suction pressure to pull the water from the hydrated colloids.

These facts are pertinent to organic acid synthesis in citrus fruits in view of the fact that large amounts of organic acids are synthesized in the leaves, and if these acids are subsequently transferred to the juice vesicles through

the albedo (mesocarp), it would appear that higher concentrations than are present should occur in the mesocarp.   The mesocarp contains sufficient cations to form salts (soluble and insoluble) as the organic acids pass through the peel to the vesicles, which contain large amounts of organic acids.   The mesocarp is notably low in amounts of free and combined organic acids, however.   During the entire growth and maturation of the fruit the passage of water and solutes from leaves to vesicles and *vice versa,* during periods of excessive leaf evaporation, did not result in accumulation of organic acids in the peel of mature fruits.   If the accumulation of citric acid in the juice vesicles is caused by its transfer from leaves to vesicles during fruit growth, the assumption can be made that there should be more citric acid in the peel and more malic acid in the juice.   According to figure 2, the leaves contain nearly three times as much malic as citric acid, and the juice of the pulp contains eight times as much citric as malic acid.   To reverse this ratio during transfer from leaves to vesicles, a conversion of malic to citric acid must take place, or citric acid must be synthesized in the vesicles.

Whatever may be the cause for this reversal in concentration of malic and citric acids, attention should be drawn to the experimental data that have accumulated to support the thesis that, under certain conditions, malic acid is converted to citric acid.   PUCHER *et al.* (12) found that the culture of tobacco leaves in the dark brought about a decrease in malic acid equivalent to 14.2%, 11.2 %, and 8.5% respectively, of the organic solids, and an increase in citric acid of 8.3%, 6.6%, and 5.1% respectively, of the organic solids.   These results were confirmed by PLATNITSKY (9), who found that citric acid in excised tobacco leaves, when exposed to solutions of potassium salts of malic, succinic, and fumaric acid, was four to five times that of the fresh leaves.

Lack of accumulation of organic acids in the peels of citrus fruits suggests that organic acids in the pulp are synthesized in the vesicles from the carbohydrates.   This deduction agrees with the limited experiments by RICEVUTO (15), who concluded that citric acid is formed in lemon fruits from reducing sugars and pentosans by enzymatic action.   Although the organic acids have not been determined for mature lemon leaves, data are available for lemon peel (19) and for lemon juice (17).   It is highly probable that the concentration of organic acids in lemon leaves is not significantly different from that in Valencia orange leaves.   The concentration of organic acids in lemon peel is about equal to that in Valencia orange peel (fig. 2).   The situation is quite different with respect to lemon juice.   A sample of mature lemon juice containing 60 mg. per milliliter of citric acid has approximately 90 m.e. of total acid per 100 gm. fresh weight.   This concentration is of the same order of magnitude as that of the leaves, but lemon peel is exceptionally low in total acid content.

Summary

The total organic acids were extracted from mature Valencia orange leaves with absolute ether, and the water solution of the extract was subse-

quently analyzed for total acid and for citric, malic, and oxalic acids. The undetermined acid fraction in the ether extract amounted to a mean of 17.76% of the total acids. This indicates the presence of one or more unknown organic acids in the leaves. The mean concentrations of citric, malic, and oxalic acids were 12.28%, 30.57%, and 39.38%, respectively, of the total acids.

An analysis of the water extract of the leaf samples showed that while most of the citric and malic acids were water soluble, most of the oxalic acid was insoluble in the water extract. Some of the calcium and all the potassium proved to be water soluble. More than half of the total calcium is combined in the leaves in an insoluble form. The insoluble oxalates are correlated with the insoluble calcium. Increase in insoluble oxalates with the growth and age of the leaves is similar to the increase in total calcium. If the sum of the water-soluble calcium and that combined with the oxalic acid be subtracted from total calcium, there remains a portion of insoluble calcium to combine with other substances (calcium pectate, *etc.*) of the leaves. The sum of the water-soluble acids and the total oxalic acid is nearly equal to the total acids of the leaves. The water-soluble organic acids and the soluble cations (calcium and potassium) form the free acid-salt relationships that contribute to the buffer capacity of the leaf sap.

The leaves contained nearly three times as much malic as citric acid, and the juice of the pulp contained eight times as much citric as malic acid. To reverse this ratio during transfer from leaves to vesicles of the pulp, it is postulated that a conversion of malic to citric acid must take place, or citric acid must be synthesized in the juice vesicles.

Possible synthesis of the organic acids in the vesicles of citrus fruits is discussed.

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STARCH HYDROLYSIS INDUCED BY POLARIZED LIGHT IN STOMATAL GUARD CELLS OF LIVING PLANTS

E. S. Semmens

It was reported in 1930 (2) that exposure to polarized light had been found to induce hydrolysis of starch grains in the mesophyll of living leaves. Similar action on starch grains *in vitro* has also been observed (1, 3, 6). The present paper gives an account of some experiments on the hydrolyzing influence of polarized light when applied to foliar stomata. In many plant forms the guard cells of leaf stomata are more easily observed in the living condition than are other starch-containing cells. Also, guard cells are specially attractive for studies on turgor or changes in relation to starch formation and starch hydrolysis, for turgor changes influence the opening and closing of the stomatal apertures. All of these tests were made with bright sky light or direct sunshine, polarized by means of a Nicol prism, or sometimes by means of reflection from a glass-covered ferrotype plate.

The list of plant forms studied included *Hyacinthus orientalis, Convallaria majalis, Pancratium fragrans, Tradescantia fluminensis, Hemerocallis fulva, Crinum americanum, Lactuca sativa, Tropaeolum polyphyllum,* and *Rheum rhaponticum.* These plants were chosen because their foliar stomata are relatively large and are essentially confined to the lower (or abaxial) leaf surface. In healthy leaves of all these forms, or in pieces of still living epidermis stripped from the leaves, polarized light was found to induce or accelerate the disintegration and hydrolysis of the starch grains of the stomatal guard cells.

Methods

For study in the living condition pieces of epidermis from the lower leaf surface were quickly placed in a thin film of water on a slide and brought to the microscope stage, or they were placed directly on the stage of the instrument. For final examination and temporary presentation such strips were killed and fixed by submerging in hot 98% ethyl alcohol for about a minute, washed in water, treated with potassium iodide-iodine solution to identify starch grains if present in the guard cells, and mounted in glycerine solution.

Results

In the following sections some representative experiments are described.

EXPERIMENT I

At 4 P.M., when the guard cells were certainly well filled with starch, a Nicol prism was closely applied to a portion of the lower surface of a leaf

of a healthy Hyacinth plant growing in a sunny window. Thus, polarized sky light reached a rectangular area (about 0.5 of an inch) of the lower leaf surface. Around that specially lighted area was a border about one-half of an inch wide, covered by the opaque frame of the prism, which thus received no direct light. While most of the lower surface received unpolarized sky light, the whole upper surface received natural sky light, some portion of which doubtless penetrated weakly through the leaf. After three days of clear weather, samples of lower epidermis were taken from the experimental leaf at about 4 P.M. and examined microscopically. It was found that the stomata of the region that had received polarized light from the prism were generally widely open, with guard cells containing very little or no starch (fig. 1A). On the other hand, in the regions that had not received polarized light the stomata were generally closed, or nearly so, and their guard cells were packed with starch grains (fig. 1B).

FIG. 1. A. Hyacinth stomata after exposure to polarized sky light. Open apertures, little or no starch in guard cells. B. Control for figure 1A, after exposure to unpolarized sky light. Narrow apertures, much starch in guard cells.

EXPERIMENT II

At about midday, a strip of lower epidermis from a healthy young Hyacinth plant in a sunny window was quickly laid out in a film of water on a slide, which was placed on a microscope stage that was provided with a substage Nicol polarizer. Thus the portion of epidermis in the field of vision was illuminated from below by polarized light only, while the rest received only natural sky light from above. Observations were made from time to time without disturbing the preparation. At first the stomata in the field were all closed or nearly so and the guard cells were well filled with starch. But within two hours the contents of the guard cells began to assume a finely granular appearance and their plastids were becoming flattened and less clearly defined. After three hours of exposure to polarized light the stomata were evidently opening and there were translucent patches in the guard cells, where the starch grains had disappeared completely. Finally, after having been fixed and stained with iodine at the end of a 4-hour experiment period, the stomatal apertures in the region that had received polarized light were found to be open, while each guard cell was

almost empty of starch, with the exception of a few grains near the cell nucleus. In the rest of the strip of epidermis, the stomata were still closed and their guard cells were full of starch, as at the beginning.

EXPERIMENT III

At about 1 P.M. two similar strips of lower foliar epidermis were taken from a healthy plant of Pancratium growing in a sunny window. The strips were quickly laid out with a little water directly on the stages of two adjacent microscopes. One of the microscopes had a substage Nicol prism, over which rested a portion of the strip. The instrument was darkened by a cover, excepting that the ocular was left uncovered for observation and bright sky light was allowed to fall on the mirror, to be reflected upward through the prism. Thus the portion of the strip in the field of vision received only polarized light from beneath, while the remainder was kept in darkness.
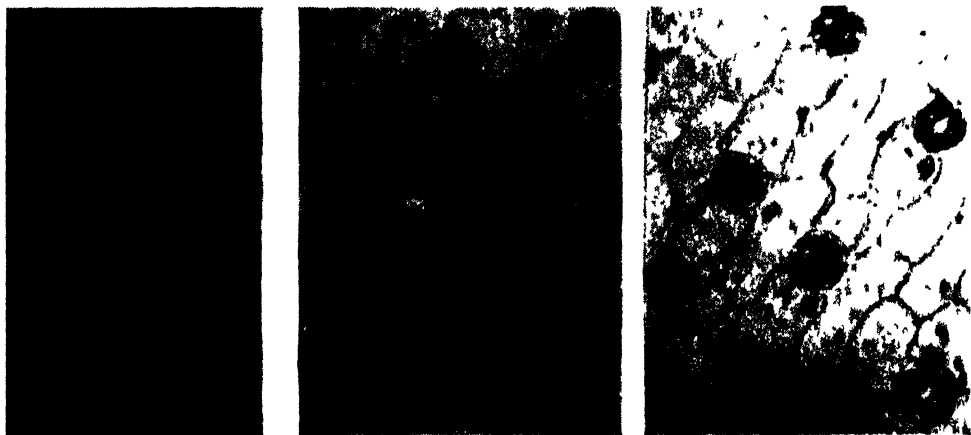
FIG. 2.   A. Pancratium stomata after exposure to polarized sky light. Open aper tures, little starch in the guard cells.   B. Control for darkness. Closed apertures, much starch in guard cells.   C. Ordinary sky light control for figure 2A.   Narrow apertures, much starch in guard cells.

The second microscope was without prism or cover and the part of its strip which was in the field was illuminated from beneath by bright sky light reflected from the mirror; the rest of this strip received only diffuse daylight from above.

Microscopic observations began at 2 P.M., when the guard cells in both fields were found to contain much starch and their stomatal apertures were closed or very narrow. By 5 P.M. the guard cells receiving polarized light were evidently losing starch and their apertures were widening, but the stomata illumined by unpolarized light from below were still essentially closed and the starch content of their guard cells was seen to have increased considerably. Both strips were fixed and stained at 6 P.M., when the stomata that had received polarized light were generally open, with their guard cells partly depleted of starch (fig. 2A). The stomata that had remained in darkness or had received only diffused light from above were closed or

nearly so, their guard cells being tightly packed with starch (fig. 2B). Those that had received bright sky light were closed or showed very narrow apertures and their guard cells were also tightly packed with starch (fig. 2C).

EXPERIMENT IV

To study the changes induced in the plastids of stomatal guard cells through exposure to polarized light, two strips of lower epidermis from a leaf of Crinum were used. One strip was laid out in a water film on a slide which rested on the stage of a microscope equipped with a substage Nicol prism. Sky light reflected from the mirror was polarized and thrown on the under side of the strip in the region over the prism, where the guard cells and their plastids could be readily examined in some detail by means of a $\frac{1}{12}$-inch ocular. As a control experiment, a similar preparation was arranged in like manner on a second microscope, which had no Nicol prism.

At the beginning of this test, a little before midday, both microscopes showed essentially the same features: stomatal apertures were closed or very narrow and each guard cell contained many (twenty or more) apparently turgid plastids full of starch. Observations were continued at intervals for about five hours, without notable changes in the stomata that were receiving ordinary light. After 2 to 3 hours the plastids of the stomata receiving polarized light were seen to have altered their positions slightly, as if in response to altered relations of pressure or viscosity. From then on, there was a gradual decrease in the size of the plastids and in their starch content, so that clear spaces appeared and broadened. Meanwhile the apertures of these stomata were opening and they were finally recorded as fully open.

At the end of this experiment, examination of the fixed and stained pieces of epidermis showed no changes in the stomata that had received ordinary light, but those that had received polarized light showed marked changes. As already noted, they were widely open. Their plastids, shrunken and apparently somewhat reduced in number, were slightly brownish in color, suggesting the iodine reaction of dextrins. Many of their starch grains had disappeared or were greatly decreased in size. The larger ones showed the usual purple iodine stain but many of the smaller grains were very indistinct and failed to show any stain at all.

EXPERIMENT V

In each of two culture chambers was placed a healthy potted plant of Tropaeolum. In the first chamber the only illumination was bright sky light reflected from a ferrotype plate covered with glass placed at an angle of 56 degrees to the horizon to produce a high degree of polarization by reflection. The second chamber was lighted by bright sky light reflected from white paper and consequently unpolarized. Tests with photographic paper indicated that light intensity was approximately equal in the two chambers in the daytime; of course both chambers were dark at night. This angle of polarization can be tested by using another Nicol prism as an analysis.

In the late afternoon of the second day of this experiment a leaf from each plant was boiled in ethyl alcohol, stained with iodine, and examined microscopically with the lower surface uppermost, without any attempt to separate the epidermis from the rest of the leaf. The findings are indicated by the photographs of figures 3A and 3B, secured by focusing on the lower epidermis, bundles and mesophyll forming the blurred background. It is evident that the guard cells of the leaf that had received polarized light from the ferrotype plate were essentially without starch (fig. 3A), while those of the leaf receiving unpolarized light from white paper were well filled with starch (fig. 3B). The iodine stain makes the stomata remarkably conspicuous in the latter photograph.
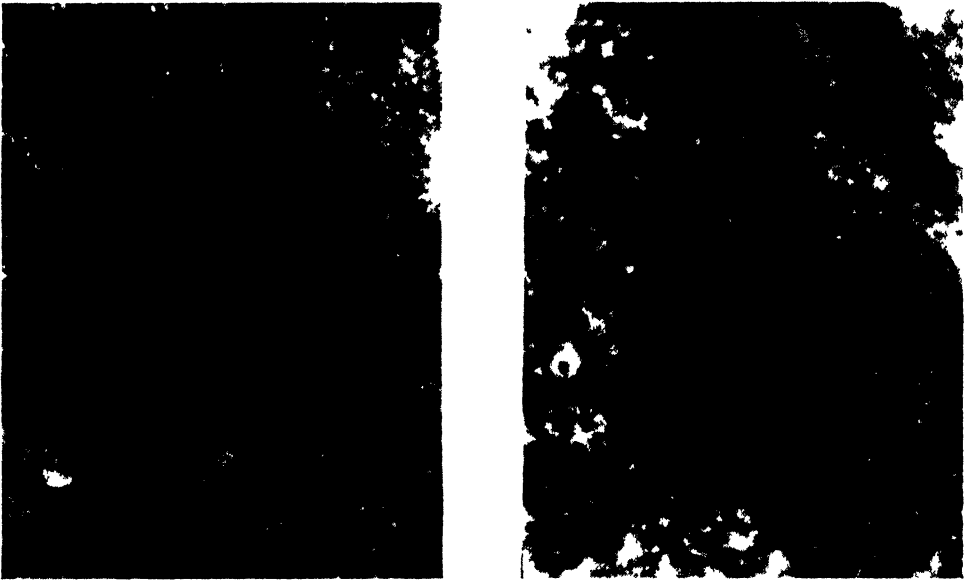
FIG. 3. A. Tropaeolum leaf surface, after exposure to polarized sky light. Apertures partly closed, guard cells with little starch. B. Diffuse sky light control for figure 3A. Apertures mostly open, guard cells with plentiful starch content.

By focusing beneath the surface of the stained inverted leaf it appeared that the mesophyll cells, which usually contain starch in Tropaeolum, were devoid of starch in both leaves. On the reasonable supposition that both guard cells and mesophyll contained starch at the start of this experiment, it appears that the mesophyll starch was hydrolyzed just as thoroughly in ordinary light as in polarized light; all light penetrating to the mesophyll must have been very weak, whether polarized or not. On the other hand, it seems clear that the polarized light absorbed in the superficial cells was sufficiently intense to induce the disappearance of whatever starch was originally present in the guard cells.

EXPERIMENT VI

At 11 A.M. on a clear day a piece of healthy Rheum leaf was placed upside down on a slide and brought under a microscope. In the magnified

field about seven stomata were visible, of which all but one were closed at that time, and all visible guard cells were well filled with starch. Fifteen minutes later a Nicol polarizer was inserted in the microscope stage beneath a part of the preparation. After 90 minutes of exposure to polarized light from the prism, four of the seven stomata first observed were well open and the remaining three had begun to open. At 2 P.M., with lower magnification and a broader field, nearly all stomata over the prism were found to be open and many of the guard cells in that region were found to be nearly or wholly devoid of starch. However, the stomata which had not received polarized light (being lighted only by ordinary diffuse light from above) were then generally completely closed, with well filled guard cells.

Experiment VII

As has been noted, when the starch of closed stomata was hydrolyzed by polarized light this process was characteristically accompanied by opening of the stomatal apertures. Opening of stomata of Crinum was specially studied by means of epidermis strips under the microscope. The apertures were nearly all closed at the start of this test, with maximal width of the most open ones being 3 to 4 microns. After a 5-hour exposure to polarized light from a substage Nicol prism almost all the stomata thus treated were wide open, with maximal apertures 8–11 microns wide.

Experiment VIII

Because the original source of light for many of these experiments was relatively weak, being sky light, used either directly or as reflected from the microscope mirror, and because the polarized light from a Nicol prism is generally only about one-half as intense as the original light entering the polarizer, it seemed desirable to make some tests with direct sunshine as original source, which would be much more intense than sky light. With this thought in mind, a young and healthy plant of Hyacinth in a south window was kept in direct sunshine, from noon till 3 : 30 P.M. on January 23, when it was safe to suppose that the guard cells would be well filled with starch. Then a Nicol prism was placed closely against the lower surface of one of the leaves, in order that the region covered would receive polarized sunshine, with full sunshine as the source. The region covered by the cork rim of the prism received no full sunshine and was almost in darkness, so far as direct light was concerned. The rest of the lower surface also received some sky light indirectly by penetration through the leaf tissues from the upper surface, but such additional radiation must have been very weak and was considered as quite negligible in the present connection. This plant stood in bright sunshine till the end of the daylight period of the 23rd, then in darkness till the beginning of the daylight period of the 24th, and finally in bright sunshine again till 4 P.M. on that day. The air temperature for this test was low, never above 50° F.

At 4 P.M. on the second day, a strip of lower epidermis was taken from

the experimental leaf; this included some of the region covered by the prism and some of that covered by the rim as well as some of the uncovered part. Microscopic examination of this strip showed that both the guard cells of the rim-darkened region and those of the uncovered region were invariably crammed with starch and appeared quite normal, the stomatal apertures being closed or very narrow (fig. 4A). In the region which had received polarized light some of the stomata were open, with little or no starch in their guard cells, but in many instances one of the guard cells had been burst open, as by an explosion [as noted in a previous investigation (5)] and its contents had been extruded (fig. 4B). In one instance the contents of a guard cell had been carried away to a distance equal to the long diameter of the cell. It appears that starch hydrolysis under the influence of rela-
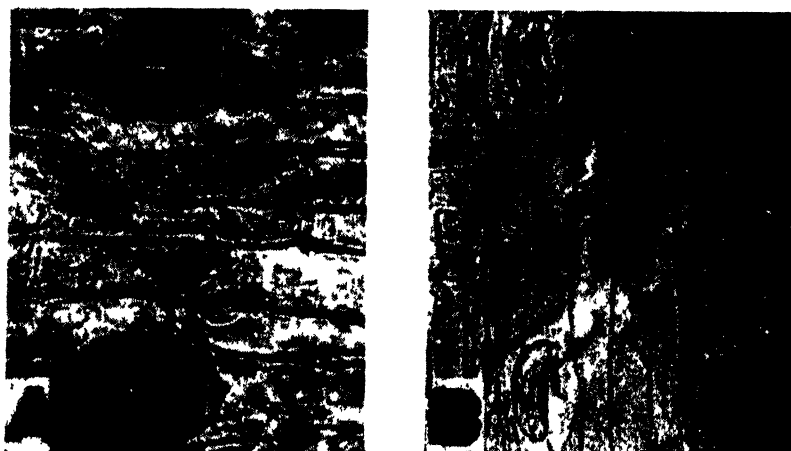
FIG. 4. A. Hyacinth stomata after exposure to unpolarized direct sunshine. Apertures nearly closed, guard cells filled with starch. B. Polarized direct sunshine control to figure 4B. Apertures widely open, guard cells with little or no starch, one exploded in each of two stomata.

tively strong polarized light had proceeded so vigorously, with accompanying rapid rise in sap concentration, that the wall had given way to the increasing strain.

EXPERIMENT IX

A Nicol prism was kept against a portion of the lower surface of a Pancratium leaf in bright sky light from May 29 to June 2. In the afternoon of the fourth day samples of lower epidermis were removed, immediately treated with boiling Fehling solution, and then examined microscopically. Only very faint brownish coloration could be detected in the guard cells that had received ordinary light, and these were well filled with starch. Guard cells that had been covered by the opaque rim of the prism showed slightly more definite brownish coloration along the margins of the aperture and they were also well filled with starch. But those guard cells that had been under the prism, and had thus received polarized light in the four daylight periods of the experiment, were found to show definite accumulation

of brown material (presumably cuprous oxide from the Fehling reaction) in otherwise translucent spaces among the nearly empty plastids. This chemical test for reducing sugar furnished additional evidence that polarized light induced hydrolysis of starch in foliar guard cells (fig. 5).
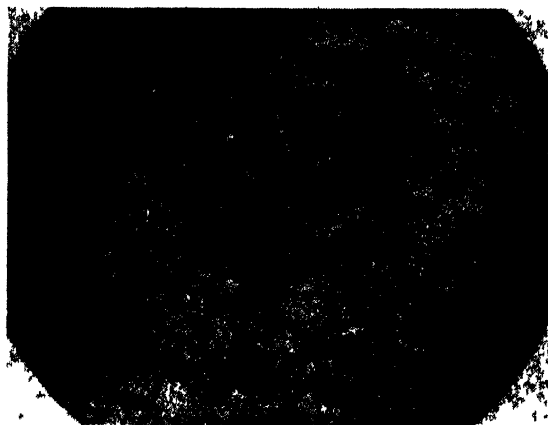
FIG. 5. Deposit of cuprous oxide in guard cells after exposure to polarized light and heated with Fehling solution.

Summary

For a number of plant forms, closed stomata of the lower foliar surface were induced to open in a few hours of exposure to polarized sky light, which also induced or accelerated the hydrolysis of starch in the guard cells. These results were obtained in experiments on uninjured leaves or strips of epidermis taken from the leaves but still alive. After several hours' exposure to polarized sky light, tests with Fehling solution indicated that decrease in starch content was accompanied by notable accumulation of reducing sugar in the guard cells. Under the influence of polarized direct sunlight, hydrolysis of starch in a guard cell was sometimes accompanied or followed by violent bursting or explosion of the cell, as if by excessive internal pressure. There was some evidence that weak polarized light which penetrated to the mesophyll cells of the leaf might induce or accelerate starch hydrolysis in these cells.

The velocity of induced hydrolysis and of the opening of previously closed stomata was naturally dependent on the intensity of the polarized light used, on the health and maturity of the plants, and their histological characteristics.

These general results, secured with a few plant forms under general conditions thus far roughly defined but clearly demonstrated (4), are sufficient to call for further more extensive and quantitative studies in this particular field of plant physiology. The present report is of pioneer or exploratory nature. It is an addition to the writer's earlier findings concerning the influence of polarized light on hydrolysis of starch in vitro (1, 3, 6) and in the mesophyll (2).

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DETERMINATION OF TOTAL AVAILABLE CARBOHYDRATES IN PLANTS

H. WEINMANN

The estimation of carbohydrates plays an important part in both pure and applied plant physiology. While satisfactory methods of extracting and determining the individual types of carbohydrates are available, the complete fractionation of these is a tedious process. Furthermore, for many purposes of a more applied nature the determination of the total available carbohydrate content is of greater significance than that of individual carbohydrates or groups of carbohydrates.

The term "total available carbohydrate" may be defined as including all those carbohydrates which can be used in the plant body as a source of energy or as building material, either directly or indirectly after having been broken down by enzymes. In most ordinary, higher green plants the bulk of available carbohydrate is composed of sugars, fructosans, dextrin and starch, whereas hemicelluloses and true cellulose act merely as structural materials and as such cannot further be utilized in the same way as the former (1, 6, 7, 8, 9, 11).

Apparently there is no satisfactory method of assessing the total available carbohydrate content by means of a single determination. Acid hydrolysis of entire samples has occasionally been used as a means of determining total "hydrolyzable" carbohydrate. The results of such determinations, however, are bound to be inaccurate and even erroneous, because it is impossible to separate the starch fraction from the structural carbohydrates in this way, and also because the relatively severe conditions of hydrolysis required for the complete breakdown of starch and dextrin result in the destruction of most of the free and combined fructose (5).

Methods

In the method described below small samples of finely-ground, air-dry plant material are subjected to digestion by takadiastase under such conditions as are necessary for the breakdown of starch, dextrin, and maltose to glucose (4). Other sugars and fructosan are extracted at the same time and, after clarification are converted to reducing sugars by suitable acid hydrolysis; after this procedure the reducing power of the neutralized hydrolysate is determined.

To prevent chemical changes during storage the samples should be killed immediately after harvesting. This can be done by autoclaving for five minutes under five pounds of pressure or, if an autoclave is not available, by some other suitable heating treatment of short duration (5). The killed material is dried in the air, or at a temperature of 40° to 60° C. When air-

dry, the material may be divided into its morphological constituents (stems, leaves, roots, rhizomes) and weighed if required. It is then ground to a fine powder and stored in air-tight bottles.

SOLUTIONS REQUIRED

BUFFER SOLUTION.—A buffer solution of pH 4.45 is prepared by mixing three volume parts of 0.2 N acetic acid with two volume parts of 0.2 N sodium acetate solution. To one liter of this solution one gram of powdered thymol is added. This dissolves slowly but almost completely. The thymol not only preserves the buffer solution indefinitely but also acts as an efficient antiseptic in the digestion of the plant material by the takadiastase (3).

TAKADIASTASE SOLUTION.—Commercial takadiastase contains a large proportion of free-reducing substances, and it is essential to remove these by means of dialysis in order to avoid undesirably high blank values. A suitable membrane may be prepared from a 4% pyroxylin solution in the following way: four grams of pyroxylin are soaked for 15 minutes in 25 ml. of absolute alcohol to which 75 ml. of ether are then added, the mixture is stirred and left standing in a closed vessel until the solution becomes clear. A large Soxhlet extraction thimble is partly filled with the solution and drained so that the liquid forms a continuous membrane on the inside of the thimble. The membrane is dried in a moderately warm place for from three to five minutes; i.e., until most of the ether has evaporated. The degree of evaporation can easily be judged by the odor. During drying the thimble is constantly rotated and occasionally blown into. The thimble with the membrane is kept in water until used.

For the dialysis five grams of commercial takadiastase are dissolved in a convenient volume of water. The solution is transferred to the thimble which is kept suspended in a jar of running tap water for three or four days. At the end of this period the dialyzed solution is filtered into a one-liter standard flask, and made to volume. Dialyzed takadiastase solutions, prepared in this way, were found to be as active as the original material while containing only traces of reducing substances. Stored in the dark, preferably in a refrigerator and with the addition of a small amount of thymol, the solution will retain its activity for approximately six weeks.

. If "undiluted" takadiastase of little or no reducing power is available, its use would make such dialysis treatment unnecessary; a 0.5% solution should be satisfactory for the purpose.

PROCEDURE

Weigh out a 0.1- to 1-gram sample of air-dry material and transfer to a 100-ml. Erlenmeyer flask which has previously been weighed accurately to the nearest 0.01 gram. The weight of sample to be used depends on the total available carbohydrate content of the material (see below under section "substrate-enzyme ratio"). Add 10 ml. of distilled water and heat for ½ hour on the boiling water-bath to gelatinize starch, inserting a small glass

funnel into the neck of the flask to minimize the loss of water. Cool to room temperature, wipe the moisture from the outside of the flask and the inside of its neck with a clean rag, place the flask on a balance, and by means of a dropping bottle add as many drops of distilled water as necessary to replace the water lost by evaporation. The counterpoise to be used must equal the weight of the flask plus the weight of the air-dry sample plus 10.0 grams. Pipette 10 ml. of buffer solution and 10 ml. of takadiastase solution into the flask, thus increasing the liquid volume of the digest to 30.0 ml. The moisture content of the air-dry sample will usually be negligible but may be taken into account if considered necessary. Stopper the flask tightly with a well-fitting rubber stopper, and incubate for 44 hours at 37° to 38° C., shaking the flask occasionally.

Cool to room temperature, add 50 to 100 mg. of powdered neutral lead acetate, shake, and allow precipitate and residue to settle. Test for completeness of the reaction with a single drop of dilute potassium oxalate solution, and filter, without washing, through a dry, highly retentive filter (such as Whatman no. 42) into a dry flask containing 100 to 200 mg. of powdered potassium oxalate. Shake, test for completeness of deleading with a drop of dilute lead acetate solution, stopper, and let stand from three to four hours, or overnight in a refrigerator.

Filter and hydrolyze a 15-ml. aliquot with 0.75 ml. of 25% hydrochloric acid for $\frac{1}{2}$ hour on the boiling water bath, attaching the flask to a reflux condenser. Cool, transfer quantitatively to a 50-ml. standard flask, nearly neutralize with 25% sodium hydroxide solution (using a few drops of methyl-red solution as indicator), and make to volume. The final extract volume (50 ml.) contains 15/30 (one-half) the total available carbohydrate of the original digest.

The reducing power of the extract may be determined by any suitable reducing sugar method. In the present experiments a previously described semimicro method using 5-ml. aliquots was employed for all determinations (10). Blank determinations should be carried out whenever a new takadiastase solution is used. For this purpose, pipette 10 ml. of distilled water, 10 ml. of buffer solution, and 10 ml. of the takadiastase solution into a 100-ml. Erlenmeyer flask. Incubate, and subsequently treat the blank digest in exactly the same way as the other digests (except for the gelatinization treatment which is omitted in the case of the blanks). Subtract the blank titration value from the titration value for the plant digest, calculate as glucose, and report as "total available carbohydrate."

A considerable number of determinations can be completed with this method in a relatively short time. The necessary equipment being available, a single worker can carry out 12 determinations simultaneously. If on each of the first three days one set of 12 determinations is started, these can be completed in succession during the following three days, making 36 determinations per week.

It is tentatively suggested that the method may also be of interest to students of problems of human nutrition. The carbohydrates determined by the described procedure are essentially those constituting that carbohydrate fraction in vegetable foods which is digestible by the human organism. The direct estimation of this fraction may well be superior to its calculation by subtraction, as is done in the conventional analysis of foodstuffs.

Experimental data

DIGESTIBILITY OF VARIOUS CARBOHYDRATES

The conditions under which takadiastase converts starch completely to glucose have been ascertained by DENNY (4). YEMM (14) used takadiastase successfully for the hydrolysis of maltose as well. A series of preliminary

TABLE I

TAKADIASTASE DIGESTIBILITY OF VARIOUS CARBOHYDRATES

CARBOHYDRATE	GLUCOSE PER DIGEST		PERCENTAGE DIGESTED BY TAKADIASTASE
	BEFORE ACID HYDROLYSIS	AFTER ACID HYDROLYSIS	
	mg.	*mg.*	*%*
Rice starch	25.4	26.6†	95.5
Maize starch (technical)	23.0	23.4†	98.3
Yellow dextrin (Merck's)	13.8	26.2†	52.7
Maltose (Schering-Kahlbaum)	24.5	25.9†	94.5
Dextrin-Maltose mixture*	25.1	26.5†	94.7
Inulin (Gurr)	0.16	24.5‡	0.65
Sucrose (Analar)	19.3	25.6‡	75.4

* Prepared by action of saliva upon rice starch; dextrin: maltose = 1: 4 (approx.).
† Hydrolyzed 20 ml. of the digest with 1.6 ml. 25% HCl for 1 hour at 15 lb. pressure.
‡ Hydrolyzed 20 ml. of the digest with 1.0 ml. 25% HCl for ¼ hour on the boiling water bath.

experiments was carried out to test the digestibility of various carbohydrates by takadiastase under the conditions proposed by DENNY (4) and subsequently adopted in the method of estimating total available carbohydrates.

Solutions of starch, dextrin, maltose, sucrose, and a suspension of inulin were prepared. The starch and dextrin solutions were boiled three minutes before being cooled and made to volume. Twenty-five mg. of carbohydrate were used in each digest, the total volume of which was adjusted to 50 ml., including 10 ml. of dialyzed takadiastase solution (corresponding to 50 mg. original takadiastase) and 20 ml. buffer solution (pH 4.45). After an incubation period of 44 hours at 37° to 38° C. the reducing power of the digests was determined and an aliquot was hydrolyzed by acid under conditions known to be necessary for the complete breakdown of the compound to hexose sugars. The reducing power of the neutralized hydrolysate was determined, and by expressing the reducing power of the digest as a percentage of the reducing power of the neutralized hydrolysate (making the

necessary corrections for dilutions and blank values), a measure of the takadiastase digestibility of these carbohydrates was obtained (table I). As will be seen, starch and maltose were almost completely hydrolyzed by takadiastase. In the case of Merck's yellow dextrin, however, only 52.7% was recovered as glucose by digestion with takadiastase. Further experiments with other commercial dextrins, all of which are produced by the action of acid upon starch, revealed that they were all more or less resistant to takadiastase (**12**). Dextrin produced from starch by saliva, on the other hand, was almost completely digestible by takadiastase. While this point needs further clarification, it may be assumed that the natural dextrins occurring in plants are likewise susceptible to takadiastase.

Inulin was found to be indigestible by takadiastase, and the new method of estimating total available carbohydrates is, therefore, not suitable for plant materials containing appreciable amounts of this carbohydrate. Inulin, which is only slightly soluble in water, will be retained in the residue and not included in the result. The partial breakdown of sucrose is doubtless due to the presence of invertase in takadiastase. As shown by previous workers, fructosans are likewise susceptible to invertase and also to takadiastase, though the action of invertase upon fructosans is very slow (**2**). It is, however, of no significance that the breakdown of sucrose and fructosans by takadiastase is incomplete, since these water-soluble carbohydrates go into solution during the process of digestion and are subsequently hydrolyzed by the treatment with acid.

EFFECT OF CLEARING AND HYDROLYSIS TREATMENTS ON SUGAR RECOVERY

In order to discover whether or not the recovery of sugars is affected by the clearing and hydrolysis treatments employed in the total available carbohydrate determination, the following series of tests was carried out: Aqueous solutions of glucose, fructose and sucrose were prepared containing approximately 40 mg. of the sugar per 100 ml. liquid volume. The latter included 35 ml. of buffer solution (pH 4.45); i.e., the same proportion of buffer solution as the digests. Solutions were subjected to the same clearing, deleading, and hydrolysis treatments as employed in the new method of determining total available carbohydrates. For the "clearing" treatment 250 mg. of powdered neutral lead acetate were used per 100 ml. solution.

In the case of fructose and glucose the reducing power of the solution was determined before treatment, after "clearing" and deleading, and again after "hydrolysis." The sucrose solution was divided into two portions, one of which was "cleared" and deleaded, while the other portion remained "uncleared."

The treatments resulted in no loss of sugar, and hydrolysis of sucrose was complete (table II). While the hydrolysis used here would appear somewhat more severe than the official methods of hydrolysis of sucrose, it should be mentioned that these experiments were carried out at an altitude

TABLE II

EFFECT OF CLEARING AND HYDROLYSIS TREATMENTS ON SUGAR RECOVERY
MILLIGRAMS SUGAR FOUND PER 100 ML. ORIGINAL SOLUTION

SUGAR	ORIGINAL SOLUTION	AFTER CLEARING AND DELEADING	AFTER HYDROLYSIS	AFTER FERMENTATION
	mg.	mg.	mg.	mg.
Glucose	43.0	43.6	43.4	0.00
Fructose	38.6	38.4	38.6	0.00
Sucrose				
"Uncleared"	41.9*	....	42.8	0.15
"Cleared"	41.9*	... ..	42.4	0.15

* Calculated value.

of 5,740 feet where the boiling point of water is not more than 93° to 94° C. The temperature reading of the acidified sugar solutions during hydrolysis was approximately 83° C. *At lower altitudes it may be necessary to modify the conditions of hydrolysis slightly to avoid loss of fructose from sucrose or fructosans.*

Aliquots of the neutralized hydrolysates were subjected to a fermentation procdure. The details of this procedure, as well as the results of the fermentation experiments, are discussed in a subsequent section.

TESTS WITH PLANT MATERIALS

SUBSTRATE-ENZYME RATIO.—In the digestion of starch by takadiastase the amount of enzyme used in relation to that of starch is an important factor. DENNY (4) reported that complete conversion of starch to glucose was ob-

TABLE III

CARBOHYDRATE RECOVERY IN RELATION TO SUBSTRATE-ENZYME RATIO*

RHIZOMES OF *Cynodon dactylon*			MIXED GRASS ROOTS		
MATERIAL USED	GLUCOSE		MATERIAL USED	GLUCOSE	
mg.	mg.	%	mg.	mg.	%
50	8.6	17.2	200	11.2	5.60
75	13.5	18.0	300	17.2	5.73
100	17.8	17.8	400	22.7	5.68
125	22.1	17.7	500	28.2	5.63
150	26.7	17.8	600	33.7	5.62
200	33.8	16.9	700	39.7	5.67
300	49.8	16.6	800	45.6	5.70
400	64.4	16.1	900	51.4	5.70
....	....	..... .	1,000	55.8	5.58
WITHOUT TAKADIASTASE					
400	16.1	4.0	500	14.4	2.88

* Glucose values expressed as milligrams per digest and as percentages of the air-dry material.

tained with a starch-takadiastase ratio of $1:2$. Whenever plant materials of unknown carbohydrate content are to be analyzed, preliminary experiments should be carried out to ascertain the most suitable sample weight to be used.

Results of two typical series are discussed. The materials used were rhizomes of *Cynodon dactylon* and mixed roots of South African grasses. A series of digestions was carried out with each of the two materials, using amounts of air-dry material ranging from 50 to 1,000 mg. per digest. The total liquid volume was 30 ml. in all cases, including 10 ml. of dialyzed takadiastase solution, corresponding to 50 mg. original takadiastase. One flask was included in each series in which the takadiastase solution was replaced by 10 ml. of distilled water. This suspension was incubated and subsequently treated in exactly the same way as the proper digests. The glucose value of this extract hence represented the total amount of water-soluble, acid-hydrolyzable carbohydrates; *i.e.*, mainly sugars and fructosans.

In the case of Cynodon rhizomes satisfactory recovery was obtained with amounts of air-dry material ranging from 75 to 150 mg. per digest (table III). Percentage glucose values resulting from these digests agreed well within the limits of experimental error, and averaged 17.8% on the air-dry basis. Higher amounts of substrate gave low values; the relatively low value obtained with 50 mg. material per digest is probably due to an analytical error resulting from the use of such a small sample. The highest sample weight with which satisfactory carbohydrate recovery could be obtained was 150 mg. of air-dry material, and the total amount of glucose yielded in this digest was 26.7 mg. Hence, carbohydrate recovery was satisfactory if the ratio of total available carbohydrate to takadiastase was not appreciably smaller than $1:2$. Since without the use of takadiastase only 4% of glucose was found, it is evident that $\dfrac{17.8 - 4.0}{17.8} \times 100 = 77.5\%$ of the total available carbohydrate was takadiastase-digestible; i.e., in the form of starch and dextrin.

The mixed grass roots were considerably lower in total available carbohydrate than were the Cynodon rhizomes and also contained less starch-dextrin in relation to total available carbohydrate, approximately 50% of the latter being extracted without the use of takadiastase. It is for these reasons that uniform results were yielded over the whole range of sample weights used; *i.e.*, up to 1,000 mg. of air-dry material. In this digest 55.8 mg. of glucose were found of which, however, only $(55.8 - 2 \times 14.4) = 27.0$ mg. were takadiastase-digestible carbohydrate, so that the actual substrate-enzyme ratio was still nearly $1:2$.

For ordinary purposes it will usually be sufficient to conduct preliminary tests with two or three different sample weights. Even the result of a single determination can be accepted as reliable if the amount of total available carbohydrate found per digest is not appreciably greater than half the amount of takadiastase used.

<center>COMPARISON OF METHODS</center>

To test the reliability of the new method, 24 samples of shoots, roots, and rhizomes of grasses were analyzed by the new procedure as well as by one of the usual methods of carbohydrate fractionation. For the latter method sugars were extracted from the air-dry, killed material with 95% alcohol; fructosans and dextrins with cold water. For the determination of starch, saliva digestion and subsequent acid hydrolysis were used. Details of these methods and percentages of the individual carbohydrates of these materials have been published elsewhere (13). In order to compare the results with those of the total available carbohydrate determination by the new method, the combined percentages of sugars, fructosans, dextrins, and starch have been calculated as glucose, and are given together with an indication of the form of the principal carbohydrate(s) in each species (table IV).

<center>TABLE IV</center>

<center>RESULTS OF CARBOHYDRATE DETERMINATIONS ON GRASSES</center>

SPECIES	PRINCIPAL CARBO-HYDRATE(S)*	TOTAL AVAILABLE CARBOHYDRATE		UNFERMENT-ABLE RESIDUE
		FRACTION-ATION METHOD	TAKADIA-STASE METHOD	
SHOOTS				
		%	%	%
Brachiaria serrata	NRS	2.42	3.14	0.00
Hyparrhenia hirta	NRS	3.14	4.30	0.27
Themeda triandra	S	4.19	5.16	0.05
Trachypogon plumosus	NRS	2.50	4.00	0.57
ROOTS				
Agrostis tenuis	F	3.57	4.05	0.00
Arrhenatherum elatius	F	4.16	4.56	0.08
Brachiaria serrata	NRS	6.59	7.06	0.07
Cynodon dactylon	ST, NRS	3.99	4.76	0.00
Digitaria trichol.	NRS	7.37	8.26	0.07
Elyonurus argenteus	S	8.97	9.62	0.57
Eragrostis chalcantha	NRS	2.90	3.74	0.03
Harpechloa falx	NRS, ST	4.48	4.91	0.00
Lolium perenne	F	3.94	4.75	0.11
Microchloa caffra	NRS	2.07	2.77	0.07
Monocymbium ceres.	NRS	4.88	5.54	0.06
Pennisetum clandest.	S, F	14.08	16.10	0.00
Phalaris arundinacea	F, NRS	5.58	6.47	0.00
Trachypogon plumosus	NRS	4.00	4.95	0.09
Tristachya hispida	ST, NRS	10.66	11.29	0.09
RHIZOMES				
Agrostis tenuis	F	19.49	20.75	0.00
Cynodon dactylon	ST, NRS	18.12	19.80	0.09
Digitaria trichol.	NRS, ST	1.98	3.14	0.04
Pennisetum clandest.	S, ST	14.10	15.42	0.00
Phalaris arundinacea	F	36.89	38.85	0.00

* Abbreviations: F = fructosans; NRS = non-reducing sugars; S = sugars; ST = starch.

A comparison of the fractionation results with those of the takadiastase method shows that the latter produced higher results than the former in all materials. LOOMIS and SHULL (5) pointed out that takadiastase may hydrolyze materials other than carbohydrates and that high values may result from the production of reducing non-sugar substances. The proportion of these can be roughly estimated by fermentation, which will remove hexose sugars from such mixtures.

All the final extracts from the takadiastase digests (including blanks) were subjected to rapid fermentation by baker's yeast, essentially according to the procedure proposed by YEMM (14). A 20-ml. aliquot of the neutralized hydrolysate and 5 ml. of a 10% suspension of washed baker's yeast were mixed in a large test tube. The mixture was incubated for four hours at approximately 35° C., a steady stream of air being drawn through. The yeast was then separated from the solution by centrifuging. From the reducing power of the solution the unfermentable residue value of the extract was calculated, making the necessary corrections for blank values and dilutions. Preliminary fermentation tests with pure sugar solutions which had been subjected to the same clearing and hydrolysis treatments as the plant digests indicated that complete or almost complete removal of sugars could be attained by this procedure (table II). It is of particular interest to note that neither the use of thymol (introduced with the buffer solution) nor that of lead acetate for clearing inhibited the fermentation.

In most of the final extracts of the plant digests mere traces of unfermentable reducing substances were present (table IV). Only one instance was found where unfermentable reducing substances could possibly account for the higher carbohydrate value of the new procedure as compared with that of the orthodox method (roots of *Elyonurus argenteus*). In this material, however, the result obtained with the takadiastase method exceeded the fractionation result by only 7%. It would thus appear that the higher values yielded by the new method are, in general, due to fermentable sugars. The low figures for the unfermentable residues exclude particularly the possibility that any appreciable amounts of hemicelluloses were broken down by the takadiastase, since this would have resulted in the production of unfermentable pentoses. The high carbohydrate values yielded by the takadiastase method must, therefore, be due to the hydrolysis of carbohydrate groups such as occur in glucosides, proteins, pectic substances, and other colloidal materials. There seems to be no *a priori* reason why such carbohydrate groups, though not determined by the more commonly employed procedures, should not be included in the fraction of physiologically available carbohydrates in the plant body.

The correlation coefficient for the results of the fractionation and the takadiastase method, as computed from the 24 pairs of determinations, is + 0.9990, indicating a positive correlation of very high significance.

That differences in the total available carbohydrate content brought about by physiological conditions can also be ascertained by the new pro-.

cedure is shown by the following series. Roots of *Tristachya hispida* which had been harvested from the plots of an experiment subjected to different clipping treatments for two seasons were analyzed (table V).

TABLE V

EFFECT OF DEFOLIATED INTENSITY ON CARBOHYDRATE CONTENT OF *Tristachya hispida* ROOTS

CUTS PER SEASON	COMBINED SUGARS-DEXTRIN-STARCH AS GLUCOSE*	TOTAL AVAILABLE CARBO-HYDRATE (TAKADIASTASE METHOD)
	%	%
1	12.97	13.93
2	11.15	12.60
4	8.60	8.37
9	1.57	1.49
16	0.95	0.97

* Sugars extracted with 80% alcohol; dextrin-starch determined on residue by saliva digestion and subsequent acid hydrolysis; fructosans absent in appreciable quantities.

Two series of experiments to assess the reproducibility of the new method were carried out. Ten determinations were done on a sample of mixed grass root material at intervals of several weeks, extending in all over half a year, thus necessitating the frequent use of separately prepared solutions of dialyzed takadiastase. The results are summarized, as follows:

<div style="text-align:center">

Mean: 7.41% total available carbohydrate

Standard Error: ± 0.05% " " "

Standard Deviation: ± 0.14% " " "

Variability Coefficient: ± 1.89% " " "

</div>

For the second test duplicate determinations were carried out on 12 samples of roots and rhizomes of grasses, the total available carbohydrate content of which ranged from 2% to 20%. Results of nine of the twelve duplicate determinations agreed within 0.25% total available carbohydrate. The standard deviation was ± 0.15% total available carbohydrate, and the coefficient of variability ± 1.80% (calculated from the deviations of the single tests from their respective means). These two figures agree remarkably well with those of the first series. The data indicate that results of the new method can generally be reproduced to within 5%, which is sufficiently accurate for ordinary biological purposes.

Summary

A new method of estimating the total available carbohydrate content of plant material in a single determination is described.

Small samples of finely ground, air-dry material are digested by takadiastase under conditions resulting in the breakdown of starch, dextrins, and maltose to glucose, while other sugars and fructosans are extracted at the same time. After clarification of the digest the latter compounds are

converted to hexose sugars by acid hydrolysis, following which the reducing power of the neutralized hydrolysate is determined.

Results obtained with the new method were higher than the combined amounts of sugars, fructosans, dextrins, and starch, when determined separately and calculated as glucose. The reducing power of the digests was, however, almost completely due to fermentable sugars, the higher values obtained by the new procedure apparently resulting from the hydrolysis of carbohydrate groups not included in the more commonly employed methods of carbohydrate fractionation. Furthermore, the results of the total available carbohydrate determination showed a positive correlation of very high significance with those of the fractionation method.

The new procedure is simple and suitable for large-scale routine analysis; results can, in general, be reproduced to well within 5%.

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INFLUENCE OF MINERAL DEFICIENCIES ON GROWTH AND COMPOSITION OF VANILLA VINES

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ARNAUD J. LOUSTALOT

(WITH FOUR FIGURES)

A root rot disease is the most important factor limiting commercial production of vanilla [*Vanilla fragrans* (Salisb.) Ames] in Puerto Rico. According to a survey of growers in 1945 (1), the disease was responsible for 40% to 50% death of vines. In 1927 the causal organism was identified as *Fusarium batatatis* var. *vanillae,* Tucker (5). The disease usually becomes more pronounced during the third or fourth year when the first heavy crop of beans is developing. Dry weather, and possibly some mineral deficiency, appear to aggravate the disease, particularly if mulch and shade are inadequate.

The purpose of the work reported here was to determine the effect of mineral deficiencies on the growth and composition of the vanilla plant, especially the effects on root growth, with a view to applying this information in the study of the root rot problem.

Materials and methods

The experiment was initiated in March, 1945 in a lime-coated greenhouse admitting about 50% sunlight. The vanilla plants were grown in 5-gallon, glazed-stone crocks containing thoroughly washed and sifted pea-size creek gravel (fig. 1). Two plants in each crock were established from 6-node cuttings of about equal vigor and girth. Each crock was equipped with a 1-inch hole at the bottom for drainage. Nutrient solutions were forced into the crocks once daily by a system of iron pipes, rubber and glass tubing, and a compressed-air electric pump, similar to the system described by KIPLINGER and LAURIE (3). The vanilla plants were trained on a bamboo lattice. Cured bamboo is hard and relatively inert, making it difficult for the vanilla to obtain nutrients through the attached aerial roots.

The full nutrient and deficiency solutions of nitrogen, phosphorus, and potassium were prepared according to HOAGLAND and ARNON (2) and renewed once every 2 weeks. Each treatment consisted of three replicates with two plants per replicate. Solution in the bottles was brought to the initial level once a day by additions of distilled water. The pH of all solutions was maintained at 6.0 by additions of weak solutions of either sulfuric acid or sodium hydroxide twice a week.

On May 6, 1946 the experiment was terminated and growth measurements, fresh and dry weight of the plants, and mineral composition of the leaves were determined.
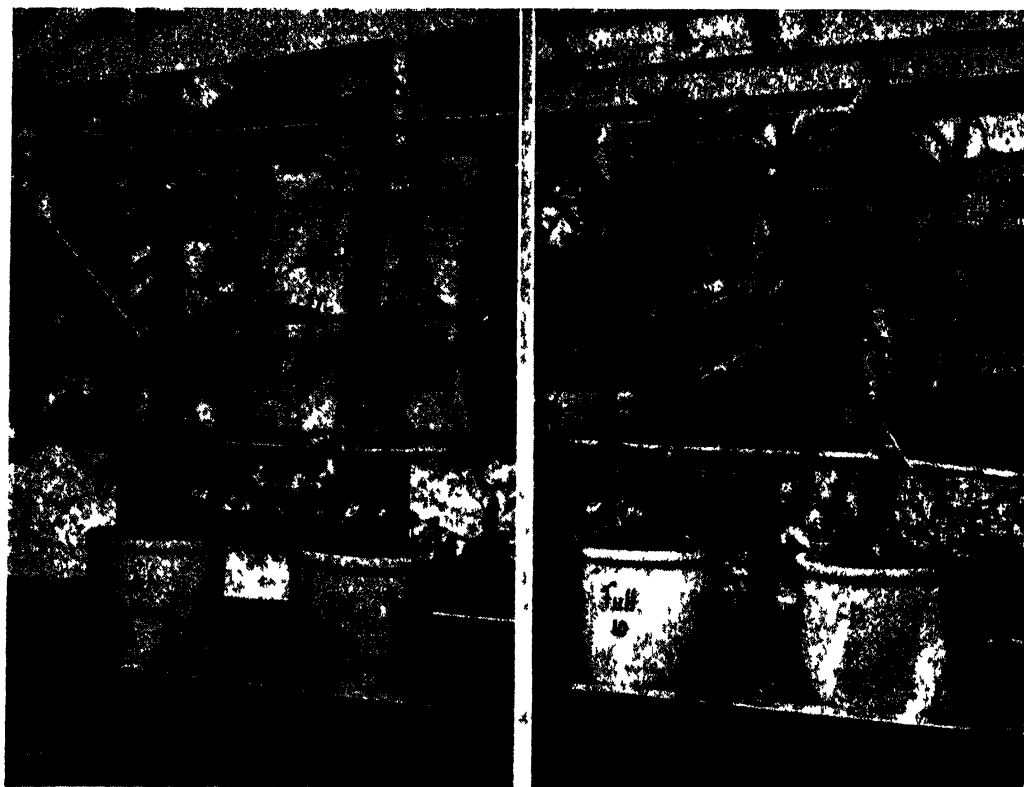
FIG. 1. Relative amount of growth of vanilla, left to right, of plants growing in Soller mulch with distilled water, in creek gravel supplied with minus nitrogen, full nutrient, and minus-phosphorus solutions.

Mineral deficiency symptoms in vanilla

NITROGEN DEFICIENCY

A deficiency of nitrogen in the nutrient solution had the greatest effect on vanilla growth. As shown in table I, the fresh and dry weights of these plants were the lowest as compared with those of plants deficient in potassium or phosphorus. Shoot growth was 64% less than that of plants receiving a full nutrient solution. Aerial root development was the poorest of all treatments. Figure 2 shows the relative girth and leaf area of the nitrogen-deficient plants.

Symptoms of nitrogen deficiency were the first to develop, appearing within about 3 weeks after the treatment was initiated. The leaves remained small and developed a yellowish green color. Although shoot growth was greatly retarded the plants as a whole showed no death of tissues. The root system, although light yellow in color and relatively sparse, appeared healthy (fig. 3). . Most of the large roots in the gravel consisted mainly of aerial roots from the original cutting which developed on the surface of the gravel adjacent to the walls of the stone crocks.

Nitrogen deficiency symptoms differ from so-called sunburn injury in the field by having an overall yellowish color on all the leaves whereas in the

case of sunburn only those leaves exposed to the direct rays of the sun show different degrees of yellowing.

PHOSPHORUS DEFICIENCY

In amount of growth, plants deficient in phosphorus were only slightly better than those deficient in nitrogen. Symptoms of phosphorus deficiency became evident about 3 months after the treatment was started. First indications of low phosphorus were a reduction in growth and a fading of green in the leaves. The leaves eventually assumed an ash-green color, with some yellow appearing before they became necrotic at the margins and tips, as shown in figure 3a. Many of these leaves shriveled, turned light brown, dried, and remained on the plant until the end of the experiment. Aerial
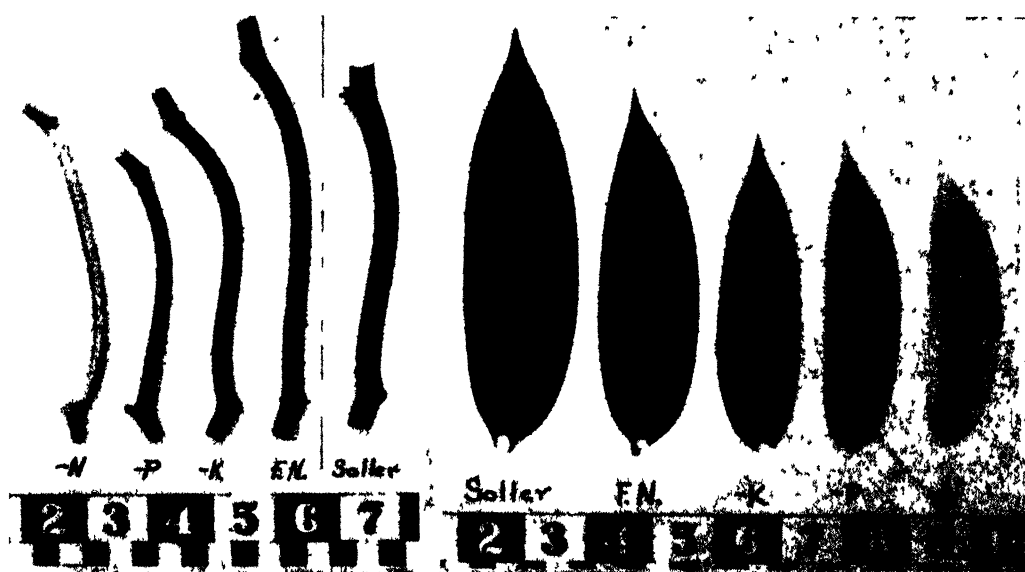
FIG. 2. Relative stem girth and leaf area of vanilla as affected by nutrient deficiencies.

root development was poor, but slightly better than that obtained with nitrogen-deficient plants.

Of particular interest in connection with the phosphorus-deficient plants is the fact that the root systems were very sparse and showed considerable dying (fig. 3a). The roots, stems, and leaves, in fact, closely resembled vanilla plants dying of root rot in the field. It is well known that Catalina soil, an extensive soil type in Puerto Rico, is low in phosphorus (4). Results obtained in this controlled experiment would indicate that more consideration should be given to supplying phosphorus to commercial vanilla, either directly to the roots as a chemical fertilizer or in the form of mulch obtained from land which has been fertilized with phosphorus.

POTASSIUM DEFICIENCY

Symptoms of potassium deficiency became apparent several weeks after those of phosphorus deficiency. As shown in table I, the fresh and dry

weights were less than those obtained from plants grown in the full nutrient solution but more than those obtained with nitrogen and phosphorus-deficient plants. The small size of the leaves and small stem girth were clear-
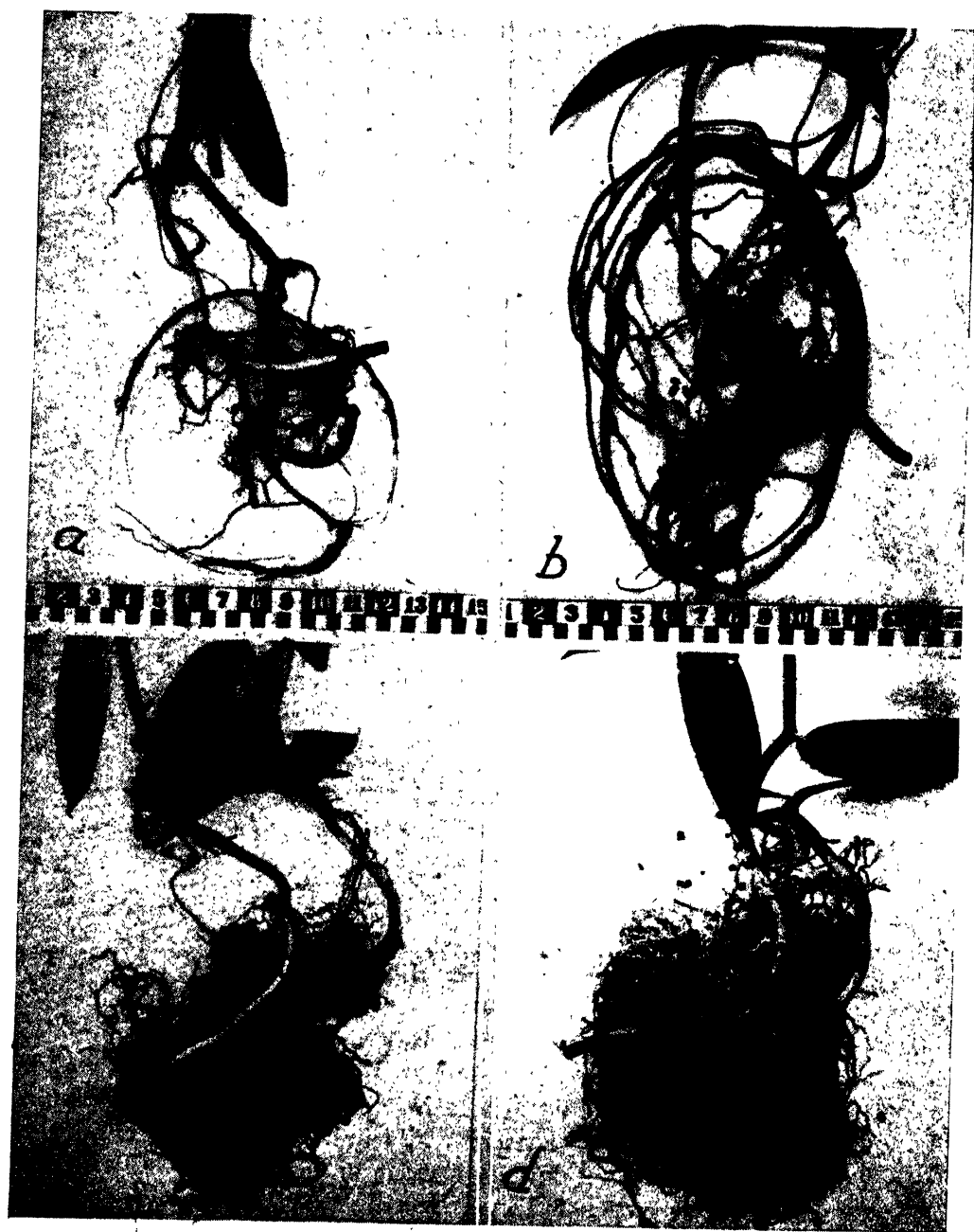
FIG. 3. Root systems of *Vanilla fragrans* Ames as affected by nutrient deficiencies of (a) phosphorus, (b) nitrogen, and (c) potassium; (d) received full nutrient solution.

cut symptoms. Also, as indicated in figure 2, the intensity of green was greatest in leaves of potassium-deficient plants. There was no marginal and tip burning of the leaves.

TABLE I

The effect of mineral deficiencies and mulch on growth of vanilla in the greenhouse

| TREATMENTS | AVERAGE WEIGHT PER PLANT | | | | | | | | MOISTURE | AVERAGE LEAF SIZE | AVERAGE GIRTH OF VINES† | AVERAGE SHOOT GROWTH PER VINE |
| | LEAVES | | STEMS | | AERIAL ROOTS* | | TOP | | | | | |
	FRESH	DRY	FRESH	DRY	FRESH	DRY	FRESH	DRY				
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	%	cm.²	mm.	in.
Full nutrient	377	29	494	46	43	10	910	85	91	39.80	8.41	362
Low calcium‡	396	30	518	48	77	12	991	90	91	38.92	8.95	359
Minus potassium	286	25	418	39	52	8	738	72	90	29.34	7.66	364
Minus phosphorus	118	9	147	15	23	4	288	28	90	25.38	6.25	180
Minus nitrogen	95	7	103	14	16	3	214	24	89	25.75	5.87	130
Catalina mulch	544	38	705	51	72	12	1321	101	92	46.21	8.50	374
Toa mulch	574	41	680	54	116	16	1370	111	92	52.29	9.41	355
Soller mulch	618	50	751	65	70	11	1439	126	91	53.37	9.50	398

* Those roots developing from new shoot growth only. Dry weights of lower root systems were lost by an unfortunate mistake. Photographic evidence, however, is shown in accompanying figures.

† An average of 3 measurements per shoot taken 6 nodes from tip, and at the middle node.

‡ These plants apparently obtained a limited amount of calcium from a few limestone pebbles discovered in creek gravel after treatment initiated.

Under soil conditions in Puerto Rico, particularly with the clay types, there has been no published evidence of potassium deficiency in plants. Small leaves and stem girth and very dark green leaves have been observed with plants growing under excessive shade in the field and in the greenhouse. It was assumed, however, that these characteristics were a result of excessive shade, although this point should be checked further.

Low calcium

Another treatment in this experiment included a set of plants receiving a solution deficient in calcium. It is evident from table I that the overall growth of these plants was as good as those receiving full nutrient solution. It was ascertained after the experiment had been in progress for several months that this was apparently due to a few limestone pebbles in the creek gravel, although no trace of calcium could be found in the nutrient solution

TABLE II

MINERAL COMPOSITION BASED ON OVEN DRY WEIGHT OF LEAVES FROM VANILLA GROWN IN THE GREENHOUSE UNDER DIFFERENT NUTRITIONAL CONDITIONS

TREATMENT	ASH	N	P	K	Ca	Mg
	%	%	%	%	%	%
Full nutrient	14.65	1.95	0.29	3.90	2.42	1.75
Minus K	14.15	2.28	0.34	0.79	4.07	2.52
Minus P	13.63	1.61	0.15	3.67	1.97	1.98
Minus N	17.59	0.58	0.37	4.30	3.36	2.58
Low Ca	15.31	1.63	0.32	3.77	1.92	2.47
Catalina mulch	15.96	2.21	0.38	3.61	2.50	2.25
Soller mulch	15.34	1.84	0.28	2.86	3.65	1.74
Toa mulch	15.63	1.98	0.54	3.98	2.09	2.69

after it had been pumped to the gravel for a period of 2 weeks. Table II shows that the calcium content of these vanilla leaves was relatively low; in fact, one-half the amount present in leaves of plants grown in Soller mulch. This would indicate that vanilla can grow satisfactorily at least for the first year, on a relatively low calcium supply.

Plants grown in mulch

Vanilla plants were grown in three types of mulch and distilled water as a supplemental study to the above experiment. From figure 4 and table I, it is apparent that the best growth was made under these conditions. Although top and root growth was definitely better for plants grown in Soller mulch, the difference was not significant. In field experiments and in commercial vanilleries, plants growing in Soller mulch have been generally better than those growing in Catalina mulch. The mineral composition of plants grown in Soller mulch (table II), does not show a marked difference in quality, except in calcium content, from that of plants grown in mulch obtained from Catalina soils. The plants grown in Toa mulch, on the other hand, were relatively high in ash and in all analyzed elements except calcium.

This was probably due to the fact that many previous fertilizer applications had been made to the soil from which this mulch was obtained.

Since vanilla apparently can obtain the necessary elements from mulch alone, the above data further emphasize the soundness of the standard recommendation in Puerto Rico to apply and maintain a heavy mulch around the vanilla roots.

MINERAL COMPOSITION OF VANILLA LEAVES

The data in table II show that the ash and potassium content of nitrogen-deficient plants was higher than that of plants in other treatments. Since nitrogen deficiency had such a retarding effect on growth and production of dry matter, it is not surprising to find high levels of minerals in these plants. It is of interest to note that although the nitrogen content of the

FIG 4. Root development of vanilla grown in mulch obtained from (a) Toa, (b) Catalina, and (c) Soller soils. Compare with figure 3.

leaves was less than one-third that in the full nutrient plants (0.58%), the vines were still alive.

The interesting feature of the mineral composition of the low-phosphorus plant was the fact that the nitrogen and calcium contents were also relatively low. Apparently phosphorus is required for the normal absorption, and, possibly the utilization, of nitrogen and calcium by the vanilla plant Phosphorus in the phosphorus-deficient plant was about one-half that in the full nutrient plant.

Potassium deficiency resulted in abnormally high calcium, nitrogen, and magnesium content. Lack of potassium in the nutrient medium apparently favors the absorption of these elements. Although the percentage of potassium in the leaves was only about one-fifth (0.79%) that of the full nutrient plant, the effect of potassium deficiency on dry weight was relatively small as compared with the effect of nitrogen and phosphorus deficiencies on dry weight (table I). The amount of phosphorus in the potassium-deficient plants was about normal (table II).

Calcium deficiency was somewhat similar to that of phosphorus in its effect on the nitrogen and potassium content of the leaves. In both phosphorus- and calcium-deficient plants the nitrogen content was relatively low while the potassium content was about normal. Magnesium was somewhat high and phosphorus about normal in calcium-deficient plants.

Although the calcium content of calcium-deficient plants was low, it apparently was not below the critical level.

In general the levels of minerals in leaves (table II) from the plants growing in the three types of mulch (table III) were as high and in most cases higher than those in plants supplied with a complete nutrient solution. Although distilled water was supplied to the mulch medium, it is evident that sufficient mineral elements were leached from the mulches to supply

TABLE III

MINERAL ANALYSIS OF MULCH USED IN GREENHOUSE VANILLA EXPERIMENT (OVEN-DRY BASIS)

SOIL FROM WHICH MULCH WAS OBTAINED	ASH	N	P	K	Ca	Mg
	%	%	%	%	%	%
Catalina	10.5	1.51	0.15	1.74	0.84	0.19
Toa	25.1	1.63	0.30	2.74	1.00	0.35
Soller	14.5	1.10	0.13	1.47	1.20	0.16

the needs of the vanilla plants. The fact, also, that the best growth was obtained with plants growing in mulch indicates that some factor or factors other than those studied may be involved.

Summary

1. A study was made of the effect of deficiencies of nitrogen, phosphorus, and potassium on root and top growth of vanilla.

2. A deficiency of phosphorus resulted in poor growth and dying of the roots and tops of vanilla. The symptoms resembled closely those frequently seen in the field on plants dying of the so-called "root rot" disease.

3. Nitrogen and potassium deficiency resulted in poor growth of the tops and roots but there was little or no death of tissues under the conditions of this experiment.

4. Good root and top development was obtained with plants growing only in mulch that was irrigated with distilled water. This indicates that vanilla can obtain the necessary minerals from mulch alone, and that the standard recommendation of heavy mulching in vanilleries is sound.

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THE EFFECT OF INDOLE-3-ACETIC ACID IN THE DIASTASE CHARCOAL MODEL SYSTEM[1]

FREDERICK G. SMITH, WILLIAM E. LANGELAND, AND ELMER STOTZ

(WITH THREE FIGURES)

A mechanism of auxin action was proposed by EYSTER (3, 4) similar to the classical adsorption theory of narcotic action of TRAUBE and WARBURG (2). It was based on the action of synthetic auxins in a model system composed of charcoal as a representative colloid, and diastase and starch as representative plant enzyme and substrate. Recent evidence (1, 5, 6, 7) that starch mobilization is one of the characteristic responses to auxin stimulation makes this proposal of increased interest and its substantiation even more urgent.

EYSTER's experiments are subject to several criticisms which are considered later, but his proposal is worthy of further examination. His conclusions were that auxins have two types of action in the model system, and presumably also in plant tissues: ". . . phase I, release of enzyme in whole or in part from charcoal; and phase II, effect on enzyme action proper" (4). He argued that in "phase I" the release of diastase or other enzyme from the inactive bound condition by auxins was the cause of growth-promoting effects and that in "phase II" inactivation of free diastase or other enzyme was the cause of growth-inhibiting effects.

The object of the present work was to reinvestigate EYSTER's observations on the diastase-charcoal model system using somewhat different and more rigorous experimental conditions. The effect of three types of interactions of auxin, charcoal, diastase, and starch on diastatic activity was investigated: (1) auxin on free diastase, (2) competitive adsorption of auxin and diastase on charcoal, and (3) the interaction of all four components.

Methods and materials

Diastatic activity was measured by saccharification rates using a reduced scale modification of REDFERN and JOHNSTON's alkaline ferricyanide technique (8). This method gives a more accurate and sensitive determination of diastase activity than is possible by starch-iodine color methods such as Eyster's. The charcoal (Norite A), diastase (Merck, medicinal U.S.P. IX), and auxin (indole-3-acetic acid, Eastman) were the same as used by Eyster and the starch was Merck's soluble starch (according to Lintner, special for diastatic power determination). The present work was restricted to indole-3-acetic acid as a typical auxin, and for the sake of

brevity it will be referred to as "IAA." All incubations and hydrolyses were carried out at 30° ± 0.2° C., and all systems containing charcoal were shaken continuously at rates sufficient to keep the charcoal evenly suspended. In the first and third types of system, hydrolyses were run in phosphate buffers while in the second type they were run at pH 5 in the standard acetate-buffered starch solutions of the REDFERN and JOHNSTON technique (8). In all cases, however, 0.5% starch was used and in most cases 0.01% diastase as in EYSTER's work. Amounts of charcoal were adjusted to the requirements of each system.

EYSTER recognized the influence of pH on diatase activity but preferred not to buffer his systems ". . . because buffer salts are not neutral in their effects on enzyme action" (4). Furthermore, it is not clear why

TABLE I

THE EFFECT OF INDOLE-3-ACETIC ACID ON ACTIVITY OF FREE DIASTASE

INDOLE-3-ACETIC ACID CONCENTRATION	DIASTASE ACTIVITY
p.p.m.	*mg.*[*]
pH 8:	
0	1.25
50	1.35
50	1.35
0	4.5
50	5.5
50	5.3
pH 6:	
0	5.3
50	5.3
50	5.3
0	5.2
50	5.4
50	5.3

[*] Maltose per minute.

he chose a pH of 8.2 at which malt diastase has only a small fraction of the activity at its optimum pH and which is outside the physiological range of most plant tissues. To insure adequate pH control and to more closely approximate physiological conditions in the present work, therefore, all solutions unless otherwise stated were buffered with 0.025 M phosphate and experiments were run at both pH 8 and pH 6. Specific conditions of the several types of experiments are given with the results where necessary.

Results

The effect of IAA on free diastase was measured by incubating the enzyme and 50 p.p.m. IAA for 20 minutes before adding the starch substrate. In no case at either pH 6 or pH 8 was any inhibiting effect observed, though at the latter pH results were more erratic in general and often showed significantly increased rates in the auxin treatments. Typical results are given (table I).

Three types of experiments were run which involved the competitive adsorption of IAA and diastase on charcoal. The purpose of these was to determine whether IAA in any way affected the amount of diastase adsorbed on charcoal at equilibrium or the rate of reaching equilibrium. The first type tested the effect of IAA on previously adsorbed diastase, EYSTER's "phase I." Amounts of diastase sufficient to saturate the charcoal were adsorbed by shaking for various lengths of time in buffers at pH 8 and pH 6. The diastase-charcoal adsorbate was separated by centrifuging, resuspended in the same buffer or buffer plus 50 p.p.m. IAA, and shaken for periods ranging from 20 minutes to 4 hours. The charcoal was then removed by filtration, the supernatant adjusted to pH 5, buffered starch

TABLE II

THE EFFECT OF INDOLE-3-ACETIC ACID IN BLOCKING THE ADSORPTION OF DIASTASE ON CHARCOAL

INDOLE-3-ACETIC ACID CONCENTRATION	DIASTASE ACTIVITY	BLOCKING*
p.p.m.	mg.†	%
pH 8:		
0	0.00	
50	0.20	3
200	0.10	1.5
0	1.10	
50	1.42	5
200	1.55	8
0	1.10	
50	1.20	1.5
200	1.60	8
pH 6:		
0	0.35	
50	0.23	0
200	0.50	2
0	0.30	
50	0.17	0
200	0.25	0
0	0.00	
50	0.10	1.5
200	0.40	4

* Blocking percentage was calculated by dividing difference in rates of charcoal systems with and without indole-3-acetic acid by difference in rates of control without charcoal and charcoal system without indole-3-acetic acid.
† Maltose per minute.

added, and the diastatic activity determined. In no case at either pH 8 or pH 6, with one possible exception, was there any greater diastastic activity in the IAA treatment than in the control. The level of activity was negligible in most cases or when measurable was probably due to small amounts of supernatant fluid wetting the charcoal in the first centrifugation. The amount of adsorbed diastase estimated by difference from that in the first supernatant fluid was such that a small percentage released could easily have been measured in the final supernatant fluid.

Since IAA seemed to cause no significant release of charcoal-bound

diastase, a search was made for evidence that it blocked or decreased the rate of diastase adsorption. Two types of experiments were carried out. In the first or blocking type, amounts of charcoal not quite sufficient to adsorb all the diastase were incubated with 50 p.p.m. or 200 p.p.m. of IAA at pH 8 or pH 6. The time required for adsorption of the IAA was determined by the disappearance of the 280 mμ indole band using a Beckman ultra-violet spectrophotometer. Diastase was then added, the mixture again incubated for a definite period, and the charcoal removed by centrifuging. The pH was adjusted to pH 5, starch added, and the diastase
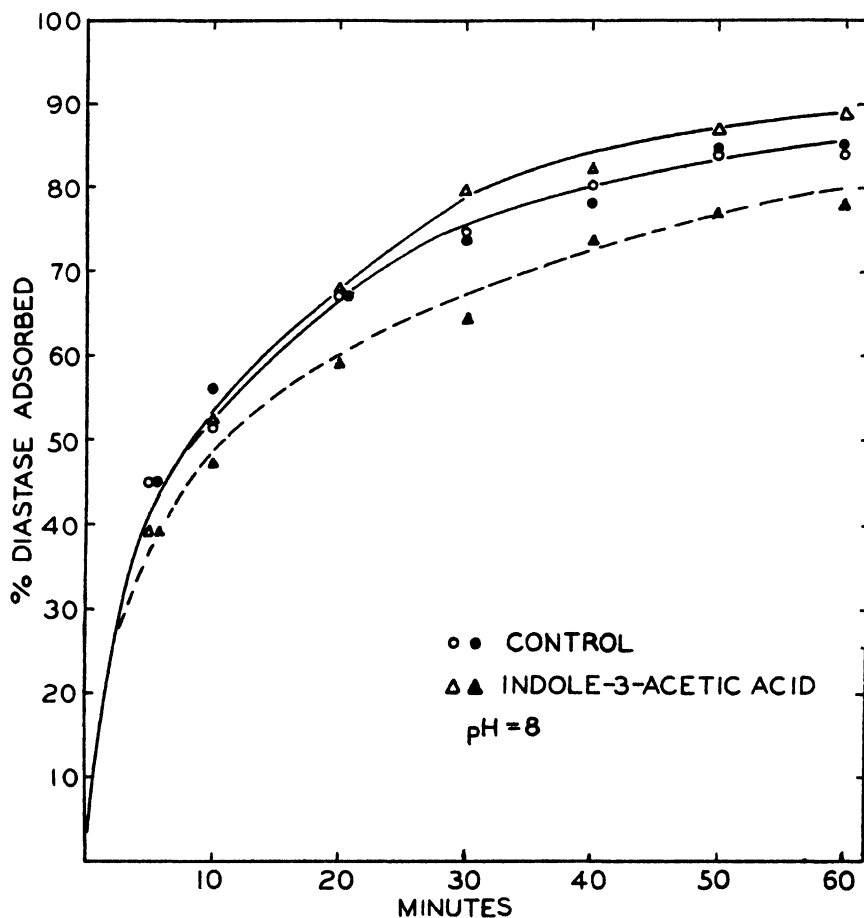
FIG. 1. The effect of indole-3-acetic acid on the rate of adsorption of diastase on charcoal at pH 8 (70 mg. charcoal in 80 ml. of 0.02% diastase and 0.025 M phosphate buffer).

activity determined. Results of representative experiments are given (table II). In no case, except a few in which the rate curves were erratic, was any large effect observed. At pH 8 there may have been significant blocking at 200 p.p.m. in a few cases.

In the second type the effect of IAA on the rate of diastase adsorption was measured as follows. A slight excess of diastase was added to aliquots of buffered charcoal suspension previously equilibrated with or without 50·

p.p.m IAA. Samples were withdrawn at intervals and added to the starch substrate. Hydrolyses were stopped after five minutes by transferring aliquots to the Na_2CO_3 part of the alkaline ferricyanide reagent. The suspension was then filtered into the ferricyanide part and the reducing power determined as usual. Results of several trials at pH 8 and pH 6 indicated a slight decrease of the adsorption rate in the presence of auxin in most cases. Typical curves are shown (figs. 1, 2). The effects, however, were probably within the experimental error of control curves which were not
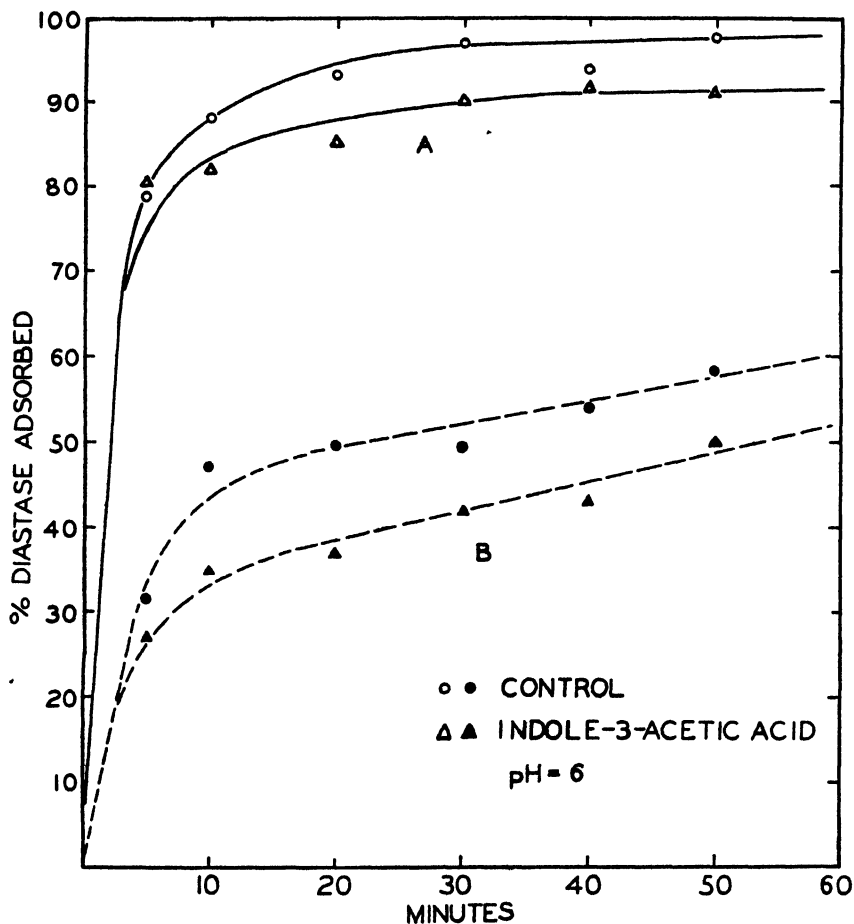
FIG. 2. The effect of indole-3-acetic acid on the rate of adsorption of diastase on charcoal at pH 6 (50 mg. charcoal in 80 ml. of diastase, curves A 0.01% and curves B 0.02%, and 0.025 M phosphate buffer).

highly reproducible. At any rate, there was no blocking effect sufficiently striking to encourage further investigation on the competitive adsorption of IAA and diastase on charcoal as a model physiological mechanism.

One further type of experiment was conducted which was similar to the one on which EYSTER seemed principally to have based his hypothesis. All components of the system were added in quick succession, the diastase last, and the diastatic activity determined at intervals. The general behavior is illustrated (fig. 3). Initial rates of the charcoal system both with and

without IAA were the same as the control without charcoal, They soon
dropped, however, becoming negligible as the diastase was withdrawn from
the hydrolyzing mixture by the charcoal. In no case did the presence of
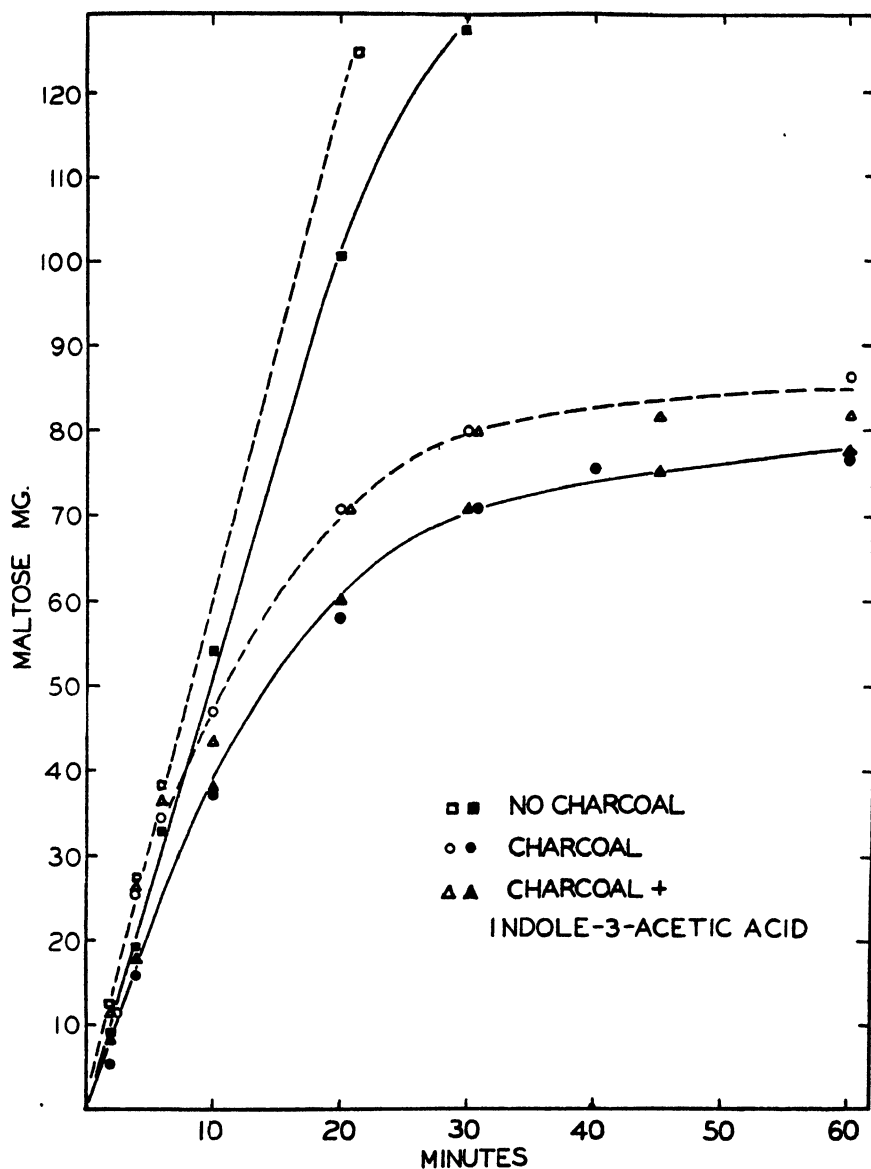
FIG. 3. The effect of interaction of indole-3-acetic acid, charcoal, diastase, and
starch on diastase activity (40 mg. charcoal in 40 ml. of 0.01% disatase, 50 p.p.m. indole-
3-acetic acid, and 0.025 M phosphate buffer at pH 6.0).

IAA significantly change the picture at either pH 8 or pH 6 though the
curves at the former pH were as usual more erratic.

Discussion

In this investigation of EYSTER's hypothesis no effort was made to repeat
his experiments exactly, first, because his methods did not lend themselves

to quantitative interpretation and, second, because the work was not reported in sufficient detail. The present results, therefore, cannot always be compared directly with EYSTER's.

Concerning "phase II" no evidence was found for inhibitory action of IAA at 50 p.p.m. on free diastase. EYSTER's own evidence for inhibition was not clear cut; the earlier report (3) [compare also SMITH (9)] was apparently based on work without pH control and the later evidence (4) seemed to consist largely of interpretations of the relative shapes of hydrolysis rate curves which depended upon a number of other factors as well. Auxins may, of course, have inhibitory action on other enzymes or even on diastase under other conditions, but the present evidence hardly provides a basis for explaining the growth-inhibiting action of auxins.

EYSTER, on the other hand, suggests that the growth-promoting action of auxins is due to the release of diastase or other enzyme from a bound form as demonstrated with the charcoal model. In the present work no evidence was found for release of charcoal-bound diastase during periods from 20 minutes to 4 hours. EYSTER's systems were allowed to stand longer periods of time but apparently were not shaken to insure adequate mixing, and no quantitative results were given. Two other types of experiments on the effect of IAA on diastase adsorption had no parallel in EYSTER's work. They provided evidence from another point of view that IAA has little if any effect on diastase adsorption on charcoal. The last type in which all components interacted together, likewise, showed no evidence of auxin effect. The proposal of EYSTER that auxins may influence growth by controlling the adsorption of enzymes was of sufficient interest to initiate the experiments reported, but no substantial support of this hypothesis was realized, at least insofar as the diastase-charcoal model system is concerned.

Summary

A reinvestigation of the action of indole-3-acetic acid in EYSTER's diastase-charcoal model system was made under more rigorous experimental conditions. No convincing evidence was found that indole-3-acetic acid either inhibited free diastase directly or markedly altered the adsorption equilibrium of diatase on charcoal.

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INFLUENCE OF PHOSPHATE ON STABILITY OF CRUDE PENICILLIN

ROBERTSON PRATT

Fermentation studies on *Penicillium notatum* in surface cultures suggested that a correlation, independent of buffer action, exists between the concentration of KH_2PO_4 initially present in the culture medium and the maintenance of a relatively high degree of antibacterial activity in the crude penicillin (8). The term "penicillin" is used here in its original sense as defined by FLEMING (6) to designate the mold-free filtrate obtained from cultures of *Penicillium* sp. This problem has been investigated further and is the subject of the present report.

The instability of penicillin in aqueous solutions, especially in certain pH ranges, is well recognized. It was shown in 1942 that maximum stability occurs between pH 5.5 and pH 7.5 (2), and more recently a thorough study of the stability of pure and partially purified penicillins in aqueous solutions at different temperatures and at different hydrion concentrations has been published by the Northern Regional Research Laboratory (3).

Concerning unpurified crude penicillin, using the term in Fleming's original sense, it was observed (1) that maximum titres were found in the medium when the pH was about 7.0. Subsequently, attempts were made to maintain a favorable pH in the fermentation medium by use of suitable phosphate buffers (5) after it had been suggested that penicillin in crude fermentation liquors may be rapidly destroyed by the biological production of excess acidity (4).

Early experiments in this laboratory suggested, however, that suitable mixtures of inorganic phosphates might exert a stabilizing effect independent of buffer action on crude penicillin, and soon after the present experiments were begun, it was suggested that a similar effect might be observed in partially purified preparations (10).

Materials and methods

Three series of nutrient solutions were prepared containing KH_2PO_4, $MgSO_4.7H_2O$, and $NaNO_3$ in different proportions but all with a total molar concentration of these three salts of 0.04 M. Solutions used for surface cultures contained in addition lactose, 0.111 M (40 gm./l.); corn steep liquor solids, 40 gm./l.;[1] and $ZnSO_4.7H_2O$, 0.00015 M (0.044 gm./l.). Those used for submerged fermentations in shaken cultures (9) contained in addition to the salts, lactose, 0.083 M (30 gm./l.), and corn steep liquor solids, 20 gm./l.[1]

[1] Corn Products Company's "Argo MVE16" and Clinton Company's steep liquor were used in different experiments. Similar results were obtained with both products.

In each series the concentration of KH_2PO_4 covered a relatively wide range. Since the initial total molarity of the solutions was constant, i.e., 0.04 M, the initial concentration of $MgSO_4$ or of $NaNO_3$ (or both) changed as the concentration of KH_2PO_4 was increased or decreased. In one group $MgSO_4$ was held constant at a low level while the concentration of $NaNO_3$ varied; in the second series $NaNO_3$ was held at a low level while $MgSO_4$ varied; and in the third lot both $MgSO_4$ and $NaNO_3$ were furnished in approximately equal concentrations in each culture, but both varied from one culture to the next (table I). Detailed discussion of the suitability of these and other solutions for supporting biosynthesis of penicillin has been published elsewhere (**8, 9**).

TABLE I

COMBINATIONS OF NUTRIENTS USED IN CULTURE MEDIA EACH WITH INITIAL TOTAL
SOLUTE CONCENTRATION OF 0.04 M

SOLUTION No.	MILLIMOLES/LITER		
	KH$_2$PO$_4$	MgSO$_4$	NaNO$_3$
Series I			
2	36	2	2
6	27	2	11
17	19	2	19
54	2	2	36
Series II			
2	36	2	2
9	27	11	2
18	19	19	2
40	8	28	4
Series III			
2	36	2	2
37	·8	16	16
58	1	18	21

Surface and submerged cultures were inoculated and incubated as previously described (**8, 9**). The crude penicillin was harvested aseptically on the seventh day and aliquots were placed immediately in an incubator at 37° C. Each filtrate was assayed daily by the standard cylinder plate method (using *Staphylococcus aureus*, NRRL 313 [F.D.A. strain 209P] as the test organism) until the potency had fallen to less than 10% of the value at the time of harvest.

Results

The experiments have been repeated several times, always with similar results. Averaged data from six experiments with surface cultures are shown (fig. 1). The percentage of initial potency remaining in each filtrate is plotted against the days of storage at 37° C. The upper set of curves is for series I in which the initial concentration of $MgSO_4.7H_2O$ was 0.002 M and that of $NaNO_3$ varied from 2 millimoles/l. to 36 millimoles/l. The center set is for series II in which the initial concentration of $NaNO_3$ was 0.002 M (0.004 M in one solution) and $MgSO_4.7H_2O$ ranged from 2 millimoles/l. to .

28 millimoles/l.   The lower set is for series III in which $MgSO_4.7H_2O$ and $NaNO_3$ both varied from one solution to the next but were of approximately equal concentration in any given culture.   For convenience, the sets of curves have been overlapped somewhat and to avoid confusion numerical
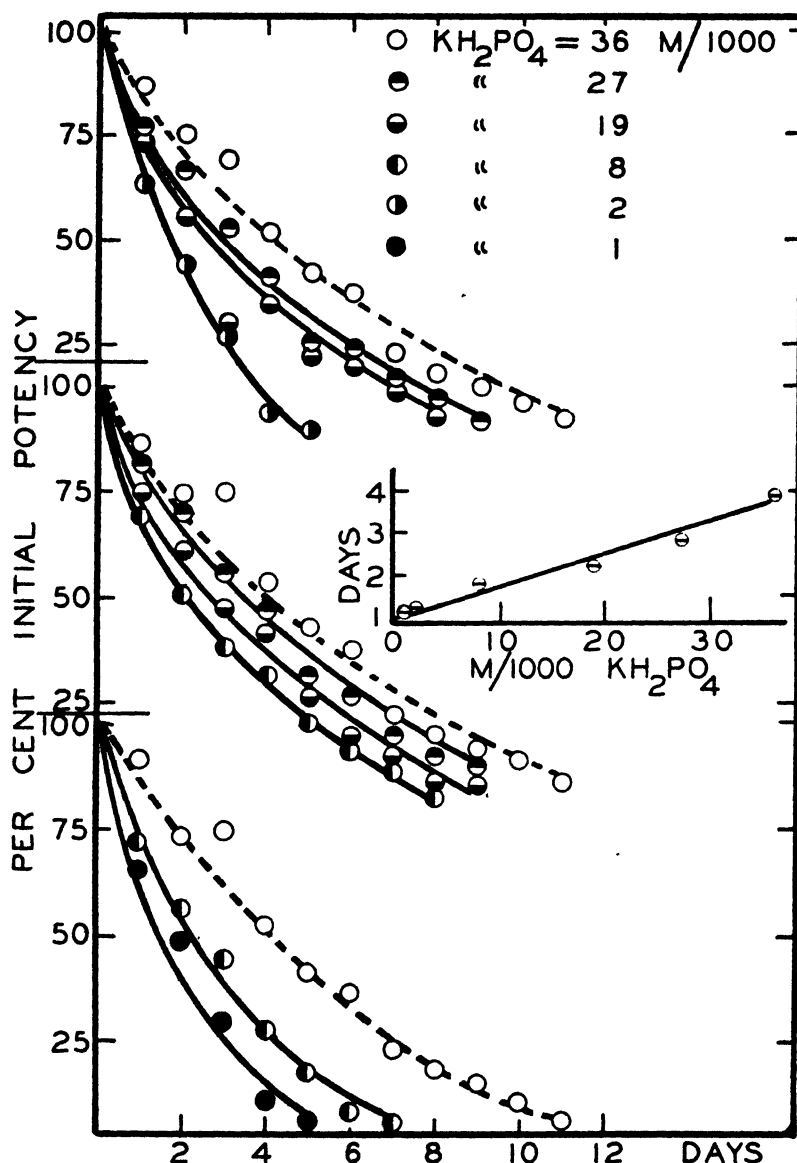
FIG. 1.   Influence of initial concentration of KH_2PO_4 in culture medium on mainte-
nance of antibacterial activity in crude penicillin stored at 37° C.   Upper set of curves
for series I, center set for series II, and lower set for series III.   (See text for further
explanation.)   *Inset:* time required for loss of 50% initial potency as a function of
concentration of KH_2PO_4 initially in medium.

values less than 25% have not been printed on the vertical axis.   The inset shows the time required for loss of 50% of the initial antibacterial potency of the filtrates as a function of the initial concentration of KH_2PO_4 in the culture medium.

Figure 2 shows similar data for crude penicillin from submerged shaken cultures with *Penicillium chrysogenum* X–1612 and *P. chrysogenum* NRRL 1984–A cultured as described previously (9). The curve for surface cultures (see inset, fig. 1) is included in this figure for comparison.

In another similar set of experiments with surface cultures phenylacetic acid (0.25 gm./l.) was added to the culture medium, and in still another set *p*-hydroxy benzoic acid (0.1 gm./l.) was added. Both of these compounds increase the yield of penicillin obtained in the crude liquor; it has been suggested that the increased yield is partly due to formation of a more stable
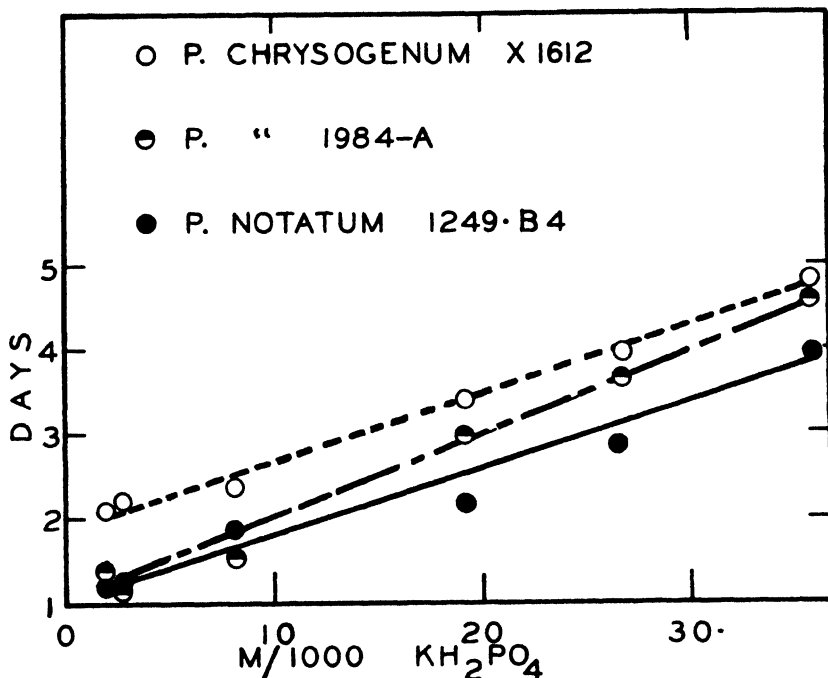
FIG. 2. Time required at 37° C. for loss of 50% of initial antibacterial potency in crude penicillin from three strains of mold as a function of initial concentration of KH₂PO₄ in culture medium.

form of penicillin, or at least to an increase in the relative concentration of such a penicillin in the mixture of antibiotics produced by the cultures.

In the present experiments, the rate of inactivation of crude penicillin from cultures with a given initial concentration of KH_2PO_4 was the same in the three sets regardless of the presence or absence of adjuvants. Curves to show the loss of antibacterial activity in solutions with high, intermediate, and low initial concentrations of KH_2PO_4 in each of the three series are shown (fig. 3). These curves are typical of those obtained in every case. Within the limits of error of the experiment, the same curve may be used to describe the data for any given initial concentration of KH_2PO_4 whether or not phenylacetic acid or *p*-hydroxy benzoic acid was added to the standard culture medium. For convenience, the curves have been overlapped and numerical values below 100 omitted. The intervals marked off represent. 25%.

Results of all the experiments show a strong correlation between initial concentration of KH_2PO_4 in the culture medium and the stability at 37° C. of the crude penicillin produced therein.   The effect was not due to buffer action, *per se,* of the phosphate ion, since the pH of all the cultures was within the range 7.2 to 7.5 at the time of harvest and did not change significantly during the period of storage.   The average unitage in the harvested liquors is shown (table II).
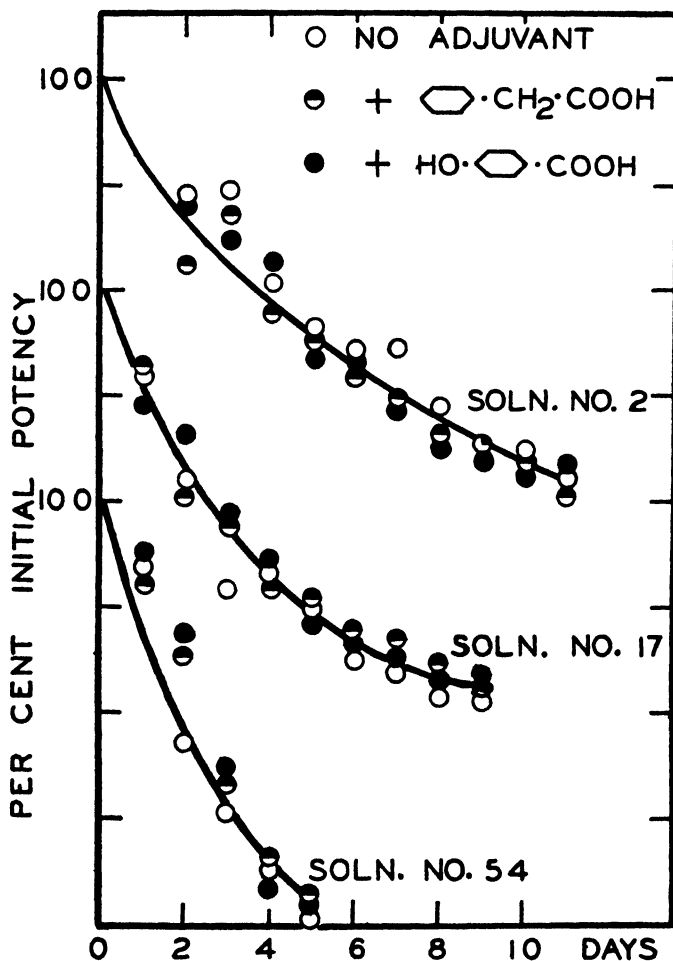
Fig. 3.   Influence of initial concentration of KH_2PO_4 and of presence of adjuvants in culture medium on maintenance of antibacterial activity in crude penicillin stored at 37° C.   Intervals on abscissa represent 25%.   (For molecular composition of solutions see text.)

The same trends are apparent in all the experiments, whether or not adjuvants were present and regardless of whether the initial concentration of $MgSO_4$ varied from low to relatively high values while that of $NaNO_3$ was uniform, or whether the initial concentration of $NaNO_3$ varied while that of $MgSO_4$ was constant.   It seems reasonable to conclude, therefore, that the variation in stability of the crude penicillin was independent not only of the concentrations of $MgSO_4$ and $NaNO_3$ but also of the presence or

absence of phenylacetic acid or p-hydroxy benzoic ,acid. In these experiments the stability appeared to be directly dependent upon the concentration of KH_2PO_4 or of phosphate present initially in the medium.

The mechanism responsible for the protective action of KH_2PO_4 or of phosphate on the crude penicillin is not clear at present, but it may well be effected through an esterification of part of the molecule similar to that reported for a number of other compounds of biological interest (7) and a consequent blocking of an enzyme system involved in the processes of destruction.

TABLE II

OXFORD UNITS/ML. AT TIME OF HARVEST IN CRUDE FILTRATES USED FOR
STABILITY STUDIES

SOLUTION No.	SURFACE CULTURES		SUBMERGED CULTURES			
	P. notatum 1249.B4		P. chrysogenum X–1612		P. chrysogenum 1984–A	
	U/ml.*	pH	U/ml.	pH	U/ml.	pH
2	136	7.25	90	7.30	120	7.42
6	95	7.10	140	7.50	81	7.32
9	145	7.20	90	7.28	80	7.40
17	165	7.30	169	7.52	80	7.39
18	90	7.18	92	7.00	93	7.19
37	153	7.32	182	7.36	78	7.32
40	152	7.26	118	7.40	106	7.41
54	126	7.28	123	7.33	77	7.33
58	131	7.42	106	7.41	118	7.42

* Addition of <===> CH_2COOH, 0.25 gm./l., increased the yields 20–25% and addition of HO – <===> $COOH$, 0.1 gm./l., increased yields 15–20%.

A forthcoming paper in the Journal of the American Pharmaceutical Association, Scientific Edition, describes parallel experiments on purified penicillin.

Summary

A study was made of the stability at 37° C. of crude penicillin from several series of cultures with different initial concentrations of KH_2PO_4. Three strains of mold were used as follows: In surface cultures; *Penicillium notatum* 1294.B4; in submerged cultures, *P. chrysogenum* X–1612 and *P. chrysogenum* 1984–A. Surface fermentations were carried out on standard media with no adjuvants and in the same media with phenylacetic acid or p-hydroxy benzoic acid added. Submerged fermentations were in the standard medium only.

In all series of cultures with each strain of mold, the results were similar—namely, the rate of loss of initial antibacterial potency varied inversely with the concentration of KH_2PO_4 initially present in the culture medium and was independent of the concentration of $MgSO_4$ and of $NaNO_3$. Presence of phenylacetic acid or of *p-hydroxy* benzoic acid in the medium did not modify the stability of the crude penicillin.

We acknowledge with appreciation the valuable assistance in the laboratory of Ruth Birch, Marjorie Decker, Iola Dunkle, Dawn Hill, Karol Hok, Luba Kellogg, and Patricia Streator.

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A VIEWPOINT FOR PLANT PHYSIOLOGY[1]

PAUL J. KRAMER

Notable as has been the growth of plant physiology and great as have been its contributions to agriculture, horticulture, forestry, and other fields, it may be questioned whether it is making as substantial contributions to plant science as it can and should make. There is considerable evidence that it is not. There are still too few courses in plant physiology offered in our colleges and some of those offered are not taught so effectively as would be possible and desirable. As a result many professional botanists and workers in applied fields of plant science have had little or no training in plant physiology. It is particularly unfortunate to find men going into agriculture and forestry lacking such training, because without it they can never really understand the growth processes of plants. There are also few plant physiologists employed in research projects by allied fields. While plant physiologists have been widely employed in horticulture for many years, few are in agronomy, and until very recently almost none in forestry. Many agricultural experiment stations do not even list plant physiologists as staff members. Likewise, few plant physiologists have entered industrial research. Although industrial laboratories such as those of food-processing companies and fertilizer manufacturers employ agronomists, chemists, plant pathologists, plant breeders, and soils specialists, they seldom have plant physiologists on their staffs. While considerable physiological research is being done in industrial laboratories, most of it is carried on under conditions such that our field receives no credit.

If it be true that plant physiology is not making so large a contribution to plant science as it is capable of making, we should search for the reasons and attempt to find remedies. One reason may be that administrators in charge of teaching and research programs do not sufficiently appreciate the usefulness of plant physiology in training students and solving problems. This seems to be largely our own fault; we have been too modest to advertise the contributions we can make and we have often failed to see or to take advantage of our opportunities.

If we are earnest in our desire for plant physiology to attain its widest possible usefulness, we must educate scientists in other fields to appreciate its possibilities. Before we start a campaign to sell plant physiology to others, however, it might be wise to consider what we have to offer. First we, ourselves, must know what plant physiology can contribute to workers in other fields of plant science. It is probable that our aims and objectives have received too little attention. Each year hundreds of papers are published *in* the field of plant physiology, but almost nothing is published *about*

the field. This is unfortunate; more consideration should be given to the objectives of our field and its proper relationship to other fields of plant science. We must develop a clearer understanding of our aims and objectives—a sort of professional viewpoint or philosophy. We need to develop a clearer understanding of the scope of our field and its relationship to other fields of plant science; this is essential to the planning of both successful teaching and productive research programs. We must know for what we are training our students if we are to know how to train them, and we must imbue them with a broad viewpoint and stimulate their imagination if they are to render maximum service.

It is difficult to define the field of plant physiology in terms of content because it is so extensive. Papers published in the area of plant physiology deal with such diverse subjects as the vitamin content of vegetables, the moisture content of leaves, the mechanism of respiration and of photosynthesis, plant hormones, weed killers, and methods of determining the permanent wilting percentage of soils. Originally the interests of plant physiologists were confined to the field of botany in its narrow sense, but in recent years they have extended to agronomy, forestry, horticulture, plant pathology, soils, biochemistry, and biophysics. Furthermore, topics once of interest solely to botanists are now important to other scientific workers. Thus the chemical composition of tobacco leaves may be as important to agronomists, soils technologists and chemists as to plant physiologists; and measurements of respiration and of photosynthesis may be as essential to the explanation of problems in forestry and horticulture as in botany. The field of plant physiology therefore can no longer be clearly distinguished from other fields by content alone; it can be better distinguished in terms of its viewpoint and relation to other fields of plant science. This is illustrated by the following diagram showing the interrelations among various factors determining the growth of plants.

Hereditary potentialities of the plant.   Environment of the plant.

The field of genetics and plant breeding.   The field of ecology and soils technology.

Internal physiological processes and conditions of the plant.

The field of plant physiology.

Plant growth.

The field of applied plant science, including agronomy, forestry, and horticulture.
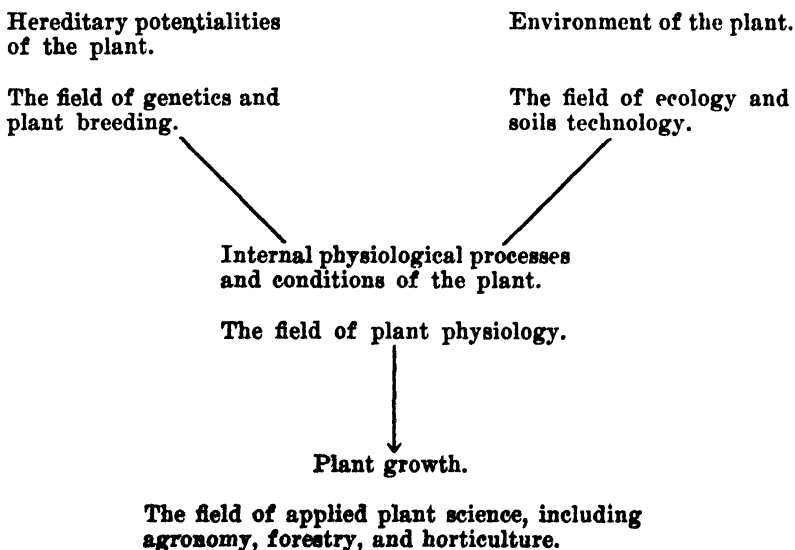
The behavior of plants, like that of other organisms, is determined by two interacting groups of factors. These are the genetic factors, determined by the heredity of the plant, and the environmental factors, determined by the conditions of soil and climate under which the plant is grown. Study of genetic factors obviously is the field of the geneticist and plant breeder, while study of the factors of the environment is primarily the field of ecologists and soils technologists.

Plant behavior, as measured in terms of quality and quantity of yield, whether corn, cotton, lumber, apples, or flowers, is primarily the concern of workers in applied fields such as agriculture, forestry, and horticulture. These men are concerned principally with the problem of how to grow larger crops of higher quality. They think and experiment chiefly in terms of crops, not of individual plants. No matter how much investigators in the applied fields learn about the effects of such specific treatments as fertilizers, irrigation, tillage, or cutting practices, on quantity and quality of growth as measured by crop yield, a very fundamental question is left unanswered. This question is: "How do variations in hereditary and environmental conditions produce differences in quantity and quality of plant growth?" The answer is, of course, that the growth behavior of an organism can only be affected through changes in its internal physiological processes and conditions. Here lies the field of activity of plant physiology. The aims of plant physiologists are to observe and measure these internal processes and conditions, to study their physicochemical mechanisms and the effect on them of variations in environmental factors, and to use the information so obtained in explaining the behavior of plants.

Plant physiologists have been effectively observing and measuring plant processes, but they have been somewhat negligent about interpreting and applying the results of their observations. Too often the interpretation and application of physiological findings have been left to workers in other fields. As a result, even though fundamental research has been done in plant physiology, full credit is seldom given for its discoveries. If plant physiology is to make its maximum contribution to plant science, plant physiologists must give more attention to the significance of their research, and especially to its usefulness in explaining plant behavior. Mere accumulation of data is not enough, no matter how carefully the data are obtained. Any industrious person can learn a technique, accumulate data, and publish a paper, but only a well-oriented investigator can interpret his results and publish a paper which makes a valuable contribution to our knowledge of plants.

Much time, money, and energy are wasted on research projects from which no worth-while results are obtained simply because the investigators do not agree on a definite objective. Funds are available for research, a certain problem seems interesting, or there is pressure to publish something; so a piece of work is done without adequate consideration of its ultimate contribution. With a little more foresight and imagination a valuable contribution might result instead of another "So what?" paper. Such a waste

of time and money is all the more unfortunate because of the tremendous amount of fundamental research so clearly needed.

There is not a single crop plant for which we have all the needed physiological data. Although much work has been done on a few species such as apples, corn, and tomatoes, there is no comprehensive, well-integrated survey of the physiological processes of any species under field conditions. Careful, and preferably simultaneous measurements of growth, photosynthesis, respiration, transpiration, stomatal opening, and other processes are needed. Such studies would make known the causes of variations in quantity and quality of yield and thereby aid in the control of the yield of crop plants. Some possibilities for worth-while physiological research in fields which have not been adequately investigated can be indicated by a few examples.

Foresters have long debated the relative importance of shading *versus* root competition for water and minerals. They have attempted to solve the problem by various types of field experiments, particularly the use of trenched plots. Their experiments were not entirely satisfactory, however, because the results could be interpreted according to the bias of the observer. Actually the relative importance of water and light in the survival of tree seedlings depends on the physiological characteristics of the competing species, hence laboratory studies of these species are essential to determine the more important factor in survival. Foresters have learned from experience that certain silvicultural practices are better than others, but they seldom know why; plant physiology should help explain in terms of physiological processes why certain practices are successful and others unsuccessful.

Plant physiologists, working for several decades in the mineral nutrition of plants, have acquired much information about the absorption mechanism and the manner in which some of these elements are used in the plant. Agronomists and horticulturists have performed numerous field experiments to study the effects of mineral elements on quantity and quality of the crop. Nevertheless, it is still not fully understood why specific fertilizer treatments produce certain results in terms of yield. This is because there is inadequate information concerning the effect of fertilizer treatments on various physiological processes. Since fertilizer treatments can affect plant growth only by changing internal physiological processes, the need for such studies seems obvious, yet little fundamental work of this sort has been done. Plant physiologists must bridge the gap between field and laboratory experimentation in order to explain plant behavior more completely.

As another example, a certain tobacco research laboratory has a remarkably complete set of chemical analyses of tobacco, showing the differences in composition of leaves grown on various soil types, with different fertilizer treatments, and in different seasons. Unfortunately, reasons for the differences in composition can only be surmised because there are no measurements of the physiological processes of tobacco plants grown under these conditions. Only after studying the water relations, the rate of photosynthesis, respiration, stomatal opening, and other processes as they vary with season, soil, and fertilization can the differences in yield and quality be explained,

and only when the causes for these differences can be explained can a rational, scientifically sound cultural program be 'developed for tobacco. The same is true of many other crops. These are clearly tasks for plant physiologists—tasks well worthy of their best efforts.

The field of plant pathology offers many opportunities for physiological research. The physiological condition of a plant has important effects on its resistance or susceptibility to disease. Furthermore, many of the abnormal conditions termed diseases are physiological in nature and could be worked on more efficiently by physiologists than by pathologists. Progress in the control of human diseases has been greatly advanced by a physiological approach, and it is probable that plant physiology can make equally worth-while contributions to the control of plant diseases. More attention should also be paid to the physiology of fungi. Mycologists have given most of their attention to the morphology and taxonomy of fungi and physiologists have concentrated on seed plants, so the physiology of fungi has not received the attention it deserves. There are scores of such opportunities to contribute to the knowledge of fundamental physiological processes and at the same time to obtain information valuable to workers in the various applied fields; the only requisite is the imagination to recognize opportunities and the training and persistence to approach them scientifically.

Two general approaches to physiological research are apparent. One is to concentrate on the details of particular processes without regard to the role of these processes in the life of the plant as a whole. Examples can be found in the investigations of the mechanisms of photosynthesis, respiration, and mineral absorption where the emphasis is on the chemical and physical processes involved rather than on their importance to the organism. The other approach might be termed "interpretative" in viewpoint because it stresses the plant as a unit and emphasizes the study of plant processes in order not merely to understand the processes but to explain the growth behavior of plants.

Greater emphasis has been placed on the interpretative or applied approach for several reasons. In the first place it seems intrinsically important to our understanding of plants, and hence for plant science as a whole, that this aspect of physiology be energetically investigated. Secondly, plant physiology as a field will flourish only to the extent that it can prove the value of its work. Under present economic and social conditions it is relatively easy to obtain funds for research projects which promise to yield results capable of practical application, while it is extremely difficult to justify large expenditures on research of purely theoretical interest. If plant physiologists insist on working chiefly on projects of theoretical importance with complete disregard for their applications, the field is apt to suffer from lack of financial support. Thirdly, development of practical applications of fundamental research usually stimulates interest in and support for the related fundamental research. Only by demonstrating that our research yields information with worth-while applications can we expect to obtain funds and support with which to continue.

For example, twenty years ago the study of photoperiodism in plants was largely of academic interest, but it was soon discovered that a knowledge of the photoperiod requirements of various crops was useful to agriculture. Appreciation of the practical importance of photoperiod has stimulated more fundamental research in this field than would ever have been possible if its practical applications had not been demonstrated. Another example of the impetus than can be given to fundamental research by practical needs is in studies of dormancy and retention of viability in seeds. Funds can often be obtained for work on species of economic importance although financial support is not usually available for work on species of no economic importance. The work on plant hormones was originally of only theoretical interest, but since it has been demonstrated that hormones and related substances have important practical applications in growing plants, research in this field has been greatly stimulated.

While emphasizing the interpretative or applied approach, the contributions which come from specialized research on the mechanisms of various physiological processes should not be minimized. More investigators with training, inclination, and equipment for work in these specialized fields are needed. Nevertheless, it seems likely that most of the support for such specialized research will come because of the need to explain problems in the applied field. Of course there is no real distinction between fundamental and applied research; there is merely a difference in viewpoint. It has often and truly been said that the more fundamental the research the wider its possible practical applications. The importance of fundamental research is widely appreciated, but more attention should be given to its application to practical problems.

This emphasis on the necessity of understanding and cooperating in the solution of problems attending the growth of economic plants does not mean that short-time applied projects of the so-called practical type are desirable; far from it. No greater disservice could be done to the applied plant sciences than for plant physiologists to forsake fundamental research on plant processes and turn to investigations of the best methods of growing corn, cotton, tobacco, or forest trees. It is important, however, that there is a tremendous amount of basic research on plant processess which should be done, because eventually this work will have important applications in growing better crops of corn, cotton, and forest trees.

Another way of expressing this viewpoint is to state that more attention should be directed to the relevancy of research and teaching to the environment of the workers. President Conant of Harvard University has been quoted as saying "To my mind a scholar's activities should have relevance to the immediate future of our civilization." The same can be said of a scientist's activities; his work should be related to the general scientific problems of his environment. Financial support can hardly be expected if the researcher insists on following his own personal interests, without regard to the rest of the world. It is possible that problems which now seem irrelevant may later become very important, but this is no excuse for neglecting

or avoiding work on problems which if successfully solved would have immediate applications.

It is obvious that this viewpoint requires a high degree of cooperation with colleagues in other fields. We must become familiar with problems in other fields of plant science and in industrial research. We need better cooperation between those workers who are principally interested in basic research and those who are chiefly concerned with applications. An exchange of ideas will stimulate imagination and broaden understanding. Many problems will be too complex to be solved by one worker or by workers in one field, hence investigators from several fields may cooperate. It will be necessary to call in chemists and physicists to aid in using the new research tools being developed in their fields.

Development of a broad viewpoint must also be taken into account in the training of students. They must learn not only of the physiological processes of plants, but also the contributions of these processes to the growth of the plant. Proper training of graduate students is of the greatest importance, for the future of plant physiology depends on our success in training future teachers and investigators.

More and more of our problems are becoming too complex for individual scientists to deal with effectively. This increases both the responsibilities and the opportunities for service of such organizations as The American Society of Plant Physiologists. The greatest service of our Society has been the publication of PLANT PHYSIOLOGY, but certain other functions are likely to become increasingly important in the future. Society committees can be very helpful because they are able to collect and evaluate information on a scale impossible to individuals.

The Society committees on chemical and physical methods have given us useful reports, and they should continue to keep us informed concerning new research methods. New committees have been authorized to study such problems as the professional status of plant physiologists, the training of students, and the relation of plant physiology to industrial research. The Society must now consider how to cooperate effectively with the other plant science societies and what its relation will be to an organization representing all the biological sciences. There is need for greater cooperation among individuals in supporting our Society and among societies in promoting objectives common to all workers in the plant sciences. Such cooperation will help us as individual scientists to work more efficiently and will enable our societies to work more effectively for us.

To be successful our officers and committees need active support. Obtaining new members and paying dues are important, but that alone is not enough to make our Society successful. All of us must study our problems and help the officers and committees solve them. Just as the success of our field depends on the value of our scientific contributions, so the success of our Society depends on the extent of our contributions as members to its activities.

DUKE UNIVERSITY
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BRIEF PAPERS

THE NON–FLOWERING CHARACTER OF SWEET POTATOES OF THE JERSEY TYPE[1]

JOHN HARTMAN

Most varieties of sweet potatoes (*Ipomoea batatas* Lam.) bloom fairly readily under proper environmental conditions. So far as the writer knows, however, there is no report in the literature of a first-hand observation of blossom formation in any variety of the Jersey group, a group defined by THOMPSON and BEATTIE (1). Physiologists, formulating theories of the fundamental mechanisms of flower formation, must take into account the unique behavior of this subspecies from a family of plants whose members are more or less characterized by their capacity to produce blossoms at most nodes while continuing to grow indeterminately. At the same time it is important that a method of producing flowers on Jersey sweet potatoes be found; it is highly desirable that Jersey varieties should be crossed with other varieties in order to combine the excellent market appearance of the Jersey storage roots with the disease resistance, high vitamin content, and vigor found in other members of the species. It is hoped that this paper may stimulate interest in the problem and result in observations and suggestions of benefit to this work.

Summary of observations and trials

Attempts to discover or induce flowering of the Jersey sweet potato have involved a long series of exploratory trials and observations. Because there was no clue regarding a means of modifying radically the normal vegetative form of growth, elaborate quantitative tests seemed inadvisable. Under different environmental conditions none of the many kinds of Jerseys has shown any marked change either in branching habit or in form of leaves. Vineland Bush, a cultivar with short internodes, and several strains of the Big Stem Jersey subgroup did not develop any more evidence of approach to a reproductive phase than did the regular Little Stem Jersey varieties. *All of the following treatments were ineffective:*

1. Continuous lighting (24-hour days) of old plants either at temperatures ranging from 50° to 60° F. or from 80° to 105° F. or higher. Light was provided by 150-watt Mazda bulbs. Some growing points were as close to the bulbs as possible without danger of burning. These plants, of course, could not be grown at the lower temperature levels. Other plants were arranged at distances up to five or six feet from the light source.

2. Varying day length with season from about 12 to 15½ hours outside and 9½ to 15½ hours in the greenhouse. Generally, natural daylight was

relied upon, but in the winter of 1943–44 sunlight in the greenhouse was supplemented by light from a battery of fluorescent lamps placed from one inch to several feet from the growing points of the various plants.   It was found that Porto Rico, Nancy Hall, Southern Queen, and many seedling sweet potatoes bloom fairly well in the spring in the greenhouse without any supplemental lighting.   Some years, Southern Queens, if widely spaced, also bloomed rather freely in the field.

3. Partial girdling of the stem by notching it to a depth two-thirds of its diameter.   Such girdling was done at different times, on old plants, and with various day lengths and light intensities.

4. Grafting young branches of Jersey sweet potatoes on weakly flowering plants of Porto Rico and of certain seedling strains.   Successful grafts are easy to make, but without continual attention the plants do not grow well.   Apparently, normal branches of the stock plant compete at a considerable advantage with the scions.   For satisfactory development it is necessary to remove all stem buds and to cut off all new shoots which continually arise from below the ground level.   The pruning done, although time-consuming, was neither sufficiently thorough nor often enough repeated.   None of the scions made more than a foot of new growth.

5. Grafting young branches of Jersey sweet potatoes on abundantly flowering plants of wild sweet potato (*Ipomoea pandurata* (L.) G. F. W. Mey) and of a wild morning glory (*Ipomoea hederacea* Jacq.).   Again, the stock plant had to be repeatedly pruned to prevent its putting out branches or new shoots.   When such care was given, many of the grafts grew exceedingly well.

6. Growing individual plants continuously in the greenhouse until they were two years old.   Such plants were kept pruned to single stems so that young branches received nitrogen through old stems.   Although generally not vigorous, they sometimes had vines 20 to 25 feet long.   Potatoes formed on these old plants were amputated on different occasions by washing away soil from around the bases of the plants and severing the connections of storage roots and main stems.   Under favorable conditions young plants of Southern Queen and of certain non-commercial strains of sweet potatoes produced flower buds on shoots not more than two months old, from time of bedding sprouted roots.   These vines were less than a foot long.

7. Wide spacing of plants in the field, for example 10 by 10 feet, and staking of principal branches.   This treatment, suggested by J. C. Miller of the Louisiana Agricultural Experiment Station, seems, under favorable conditions of soil moisture and atmospheric humidity, to encourage the blossoming of some varieties, *e.g.*, Porto Rico.   Even with girdling, however, wide spacing was entirely ineffective for Jersey sweet potatoes.

8. Starving plants for nitrogen both in the field and in the greenhouse.

Attempts to induce flowering have extended over a period of eight years. Various combinations of the eight treatments listed above have been used and numerous environmental conditions have been employed.

The writer is indebted to several persons who assisted in the various exploratory trials: Miss FRANCES SHOAF, MR. ERNEST NORDLINGER, and especially MISS MARY BRUCE, a graduate student who has been very successful both in making grafts of sweet potato on *I. pandurata* and *I. hederacea* and in maintaining the growth of scions.

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1. THOMPSON, H. C., and BEATTIE, J. H.   Classification and variety descriptions of American varieties of sweet potatoes.   U. S. Dept. Agr. Bull. **1021**.   1922.

THE CHEMICAL RELATION OF THE LEAF, SHEATH, AND STEM OF BLUE PANICUM, *PANICUM ANTIDOTALE* RETZ.

MARTIN GIBBS

It has been the custom in this laboratory in preparing grasses for chemical analysis to separate the tops into leaves and "stems" by clipping the leaves next to the ligule; thus the "stem" consisted of the true stem plus the leaf sheaths. The separation was made in this way because of the difficulty of removing the sheath from the stem; however, from a structural point of view, the sheath belongs with the leaf. It was of interest, therefore, to compare the leaf blade, sheath, and stem on the basis of chemical constituents; no reference to work of this sort has been found in the literature.

Procedure

Blue panicum, *Panicum antidotale* Retz., which had been grown in the greenhouse was harvested when the plants were fully mature and about three feet tall. The plants, separated into stems, leaf blades, and sheaths dried rapidly in an oven ventilated by a stream of air at 70° C. The tissue was ground in a Wiley mill to pass a 40-mesh screen.

Data on the comparison of chemical constituents of leaf, sheath, and stem are shown (table I). Total nitrogen was determined by a modified micro-Kjeldahl method as described by NOGGLE (3). The ash components were determined on a wet-ashed sample prepared and analyzed as described by NOGGLE (2).

Reducing and total sugars were determined on a one-gram sample with the Shaffer-Somogyi reagent according to HEINZE and MURNEEK (1). The carbohydrate results are reported in terms of glucose. All analytical results are reported as milligrams of the chemical constituent per gram of dry tissue.

Results and summary

The values in parentheses have been obtained on the basis of the sheath value as equal to 100. There are significant differences in the chemical constituents of the leaf blade, sheath, and stem of blue panicum (table I). These differences justify the separation of grass shoot samples for chemical analysis into the three groups used in this study. It would also be possible to separate the samples into two groups, one consisting of the leaf blades plus sheaths and the other consisting of the stem only; this method appears more advantageous than the older way of separating samples into one group of leaf blades only and a second group of stem plus sheaths.

The author is indebted to DR. G. R. NOGGLE, Assistant Professor of Botany, University of Virginia, for suggesting this problem.

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TABLE I

COMPARISON OF CHEMICAL CONSTITUENTS OF LEAF, SHEATH, AND STEM OF BLUE PANICUM

PLANT PART	REDUCING SUGAR	TOTAL SUGAR	MAGNESIUM	PHOSPHORUS	POTASSIUM	NITROGEN	CALCIUM
	mg./gm.	mg./gm.	mg./gm.	mg./gm.	mg./gm.	mg./gm.	mg./gm.
Leaf blade	1.04 (122)	1.39 (83)	1.19 (89)	2.84 (105)	26.6 (90)	14.6 (239)	13.6 (356)
Sheath	0.85 (100)	1.67 (100)	1.34 (100)	2.70 (100)	29.5 (100)	6.1 (100)	3.8 (100)
Stem	2.23 (262)	3.68 (220)	0.58 (45)	3.14 (116)	12.2 (41)	2.9 (48)	1.4 (36)

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1. HEINZE, P. H., and MURNEEK, A. E.   Comparative accuracy and efficiency in determination of carbohydrates in plant material.   Univ. of Missouri Res. Bull. **314**.   1940.

2. NOGGLE, G. R.   Applications of spectrophotometric methods to the problems of chemical analysis in the study of plant physiology.   Illinois Acad. Sci. Trans. **38**: 64–67.   1945.

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A MODIFICATION OF THE CELLULOID HOLDER
FOR COBALT CHLORIDE PAPER

VICTOR A. GREULACH

The celluloid holder for cobalt chloride paper devised by MEYER (2) is a device generally satisfactory for use in studies of water vapor loss from leaves, but the gummed eyelet reinforcements used for attaching the paper to the celluloid often do not adhere well. This difficulty can be overcome by using adhesive cellulose tape (Scotch Tape) instead.

A piece of half-inch tape about one and one-half inches long is torn off and a hole approximately one-fourth inch in diameter punched in the center. A square or disc of cobalt chloride paper about five-sixteenths of an inch in diameter is placed over the hole on the adhesive side of the tape, the tape attached to the celluloid strip, and the projecting ends of the tape trimmed even with the edges of the strip. It is not necessary to notch the celluloid strip as is done when the gummed eyelet reinforcements are used.

If desired the size of the holder may be increased slightly allowing permanent color standards as described by LIVINGSTON and SHREEVE (1) to be attached to the hygrometric paper. This is not feasible when gummed eyelets are used.

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1. LIVINGSTON, B. E., and SHREEVE, E. B. Improvements in the method for determining the transpiring power of plant surfaces by hygrometric paper. Plant World **19**: 287–309. 1916.
2. MEYER, B. S. The measurement of the rate of water-vapor loss from leaves under standard conditions. Amer. Jour. Bot. **14**: 582–591. 1927.

NOTES

Election Results.—The retiring Secretary has announced the following results in the annual election of officers: President, F. W. WENT; Vice-President, R. B. WITHROW; Secretary, C. H. WADLEIGH; Executive Committee, P. J. KRAMER; Editorial Board, D. R. HOAGLAND. The Constitutional Amendment passed with only one dissenting vote. The vote for the time of meeting stood 154 for September to 175 for December.

Life Membership.—The Executive Secretary-Treasurer announces the purchase of a Life Membership by ROBERT E. BURNS, Department of Botany, State University of Iowa, Iowa City, Iowa. An earlier purchaser was DR. WENDELL MULLISON of Midland, Michigan.

Dr. C. Stacy French.—DR. C. STACY FRENCH, Associate Professor of Plant Physiology, at the University of Minnesota, Minneapolis, has been appointed to the Division of Plant Biology of the Carnegie Institution of Washington at Stanford University, California, as announced by the Institution on March 28. DR. FRENCH has assumed the duties of Director of the Division, as of July 1, succeeding DR. H. A. SPOEHR, who is relinquishing administrative responsibilities in order to devote his full energies to his researches on the products of photosynthesis. DR. SPOEHR has served as Chairman of the Division since 1932.

Born in Lowell, Mass., December 13, 1907, DR. FRENCH was graduated from Harvard in 1930, and received the degree of Ph.D. from that university in 1934. He was a research fellow at the California Institute of Technology in the following academic year. After spending a year working with PROFESSOR OTTO WARBURG at the Kaiser Wilhelm Institut für Zellphysiologie in Berlin, DR. FRENCH returned to Harvard as Austin teaching fellow in biochemistry for 1936–38. He was instructor in chemistry at the University of Chicago from 1938 to 1941, when he joined the University of Minnesota faculty.

In his researches, DR. FRENCH has been concerned especially with such subjects as cellular respiration, and the photosynthesis of purple bacteria and of green leaves. His work is thus closely related to the general programs of the Division of Plant Biology. This research center was organized in its present form by the Carnegie Institution of Washington in 1928, its history dating back to 1903, when the Institution's Desert Laboratory was established. Its major fields of work are the biochemistry of plant life, in which photosynthesis is of primary importance, and experimental taxonomy, in which the forces controlling the natural evolutionary process are the main focus of interest.

DR. SPOEHR, who joined the staff of the Desert Laboratory in 1910, became chairman of the Division in 1927. After serving as director for the natural sciences of the Rockefeller Foundation in 1930–31, he returned to

the chairmanship of the Division. Carbohydrate metabolism and photosynthesis are his major fields of investigation, concerning which he has published many scientific papers. He will continue his investigations on the production of carbon compounds by plants.

Trace Elements in Plants and Animals.—WALTER STILES. The Macmillan Company, New York. 1946. 189 pages. $2.75.

The author presents a digest of experimental work on trace elements in the life of plants and animals carried out in both field and laboratory. In this book a highly technical subject is presented in an understandable manner and brings together in one volume information of value for the technical research worker. This work should be valuable to all persons interested in the nutrition of plants and animals. (Fertilizer Review.)

Elements of Soil Conservation.—HUGH HAMMOND BENNETT. McGraw-Hill Book Co., Inc., New York and London. 1947. 406 pages. $3.20.

The author has drawn upon many years of experience to present a comprehensive text on methods and techniques involved in modern soil conservation. It should prove valuable as a text for agricultural students. The author points out many practical aspects of the problem and presents striking figures to illustrate his points. (Fertilizer Review.)

The Production of Tobacco.—WIGHTMAN W. GARNER. The Blakiston Co., Philadelphia. 1946. 516 pages. $4.50.

Dr. Garner has produced an authoritative and well-illustrated book covering all phases of tobacco production. Two chapters are devoted to fertilization and mineral nutrition. The book is of special value for student and reference use with complete bibliographies. (Fertilizer Review.)

Fifty Tropical Fruits of Nassau.—KENDAL and JULIA MORTON. Text House, Coral Gables, Florida. $3.50.

While this book was written to acquaint visitors with the assortment and variety of the fruits of Nassau, it is well recommended by qualified botanists. The volume is richly illustrated and of the narrative-inventory type. Photographs of the fruits are quite complete and the text deals primarily with the history, description, and utility of the fruits, including the manner of preparation for the table.

The Cereal Rusts as Exemplified by the Leaf Rust of Wheat.—K. STARR CHESTER. Chronica Botanica, Waltham, Mass. 1946. 270 pages. $5.00.

The principles developed in this monographic treatment of wheat rust apply to cereal rusts in general as well as plant diseases in general. The book is based on the world literature and the author's researches in this field. Particular emphasis is given to the effect of environment on rust and

host plant, host-parasite relationships, rust dissemination, specialization, and control.

Index to the Literature on Photochemical Analysis.—American Society for Testing Materials, 1916 Race St., Philadelphia 3, Pa.   200 pages. $3.00.

This Society, through its Committee E–2 on Spectrographic Analysis, has sponsored the publication of two indexes on this subject.   One, issued in 1940, covered the years from 1920 to 1939; the second, just available in 1947, covers the period from 1940 to 1945, inclusive.   The second Index has almost a thousand contributions to literature in this field with brief abstracts of the material.   The painstaking and intensive work of maintaining and compiling these indexes was carried out under the direction of staff members of the National Bureau of Standards.   Copies may be obtained from the A.S.T.M. Headquarters.

Enzymes.—JAMES B. SUMNER and G. FRED SOMERS.   Second Edition. The Academic Press, Inc., New York.   380 pages.   1947. $6.50.

A revised and enlarged second edition with new material and complete revision of some sections.   The preparation of various enzymes is stressed and methods are given for their extraction and purification.   The first chapter of the book is concerned with the general properties of enzymes and this is followed by detailed treatment of the hydrolytic and oxidative enzymes.   A short discussion of desmolases, hydrases, and mutases is then presented with the addition of one chapter on carbohydrate metabolism.   Each chapter is followed by a complete bibliography of abbreviated citations. This new edition should be of interest as a general reference and represents a usable compilation for the laboratory.

DISTRIBUTION OF RUBBER AND RESINS IN GUAYULE

O. F. CURTIS, JR.

(WITH ONE FIGURE)

Introduction

Rubber contents are so frequently the focus of attention in investigations of guayule (*Parthenium argentatum* Gray) that an account of rubber distribution in the plant may contribute to experimental studies as well as to purely descriptive literature concerning the plant. In the choice of plant material for study or for sampling, or in the interpretation of results, the relative amounts and concentrations of rubber in the various parts may be a pertinent factor. Even commercial scale harvesting and milling operations are aided to some degree by knowledge of which plant parts contribute significantly to rubber yields and which are insignificant or mere diluents.

The pattern of rubber distribution as revealed qualitatively under the microscope has been thoroughly studied and described by Ross (8), LLOYD (6) and ARTSCHWAGER (1). In plants older than one year, the vascular rays of phloem and xylem carry the major portion of the rubber. Other parenchymatous tissues, the pith, primary cortex, epithelial cells of the resin canals, and xylem parenchyma contain rubber; but, partly because they comprise a smaller fraction of the plant, the amount of rubber they contribute is less important. In younger plants, some of these latter tissues contribute a relatively larger part of the total rubber. Rubber is evident in the leaf parenchyma, but only in very small amounts.

Only incomplete information is to be found concerning the macro-, but quantitative distribution of rubber in guayule. Whittlesey (13) separated several portions from native plants of uncertain but evidently advanced age. As determined by carbon tetrachloride extraction, following extraction with alcohol and water, the bark of the root and that of the trunk yielded approximately 20% rubber. Practically no rubber was obtained from the wood of these parts. This supported an impression, already current at that time, that the rubber in guayule is largely restricted to the bark. Above the trunk, which was apparently the sole remnant of the basal branching system typical

of a vigorous young cultivated plant, the leafy branches were analyzed as a composite sample. Aside from Whittlesey's report the literature offers little but fragmentary information, rarely accompanied by published data. Unpublished data from a preliminary survey of a few plants by H. P. Traub and B. J. Cooil indicated a general distribution between bark and wood and between root and top not unlike that shown in the following pages.

Olson[1] separated plants into top and basal portions. From a series of such separations in which the division was at various levels, he obtained indications that the maximum concentration of rubber was in the lowermost (proximal) parts of the branches. This obtained for recovery by milling procedures as well as by direct chemical analyses, and the quality of rubber yielded also seemed better in the lower parts of the branches than in either the root portions or the youngest upper branches.

Materials and methods

Plant material was obtained from various plantations and experimental areas of the Emergency Rubber Project in the vicinity of Salinas, California. The history of the plants prior to field culture was that of the usual planting procedure. Seedlings produced in thickly planted, irrigated nursery beds were transplanted to the field in the fall or winter, after less than a year of growth from seed. The indicated age of the plants refers to the years of growth in the field following transplanting, although the oldest tissues in the root and crown, which were carried over in the transplant, were of course nearly a full year older. Five plants were combined to provide the material for each separation, and the recorded values are the means of six such groups.

The general pattern of subdivision and designation of parts is illustrated (fig. 1). The root system was detached where constriction begins below the swollen crown portion. The short lengths of secondary and finer roots obtained as the plants were dug were removed as a separate sample of branch roots. This represents only a minute part of an extensive branching root system (7), but perhaps more than is removed in the normal harvesting operation of undercutting. The primary root, devoid of the lateral roots, represents approximately the root of the original transplant. An arbitrary decision placed the upper limit of the crown at the level where the few enlarged branch bases gave rise to the more profuse main branching system. Usually the plane of the soil surface passed through the crown region. Above the crown, the branches were separated into the stem lengths corresponding to the successive annual increments of growth. The designations "first year stem, second year stem," etc., accordingly indicate the year of growth after transplanting in which the primary tissues of the stem segments were formed. Detection of the successive stem segments is made possible by the shortened internodes which appear as cessation of bud growth occurs in

[1] OLSON, D. S. Distribution and quality of rubber milled from plant fractions. Unpublished report in the files of the Emergency Rubber Project, Salinas, California. July 14, 1944.
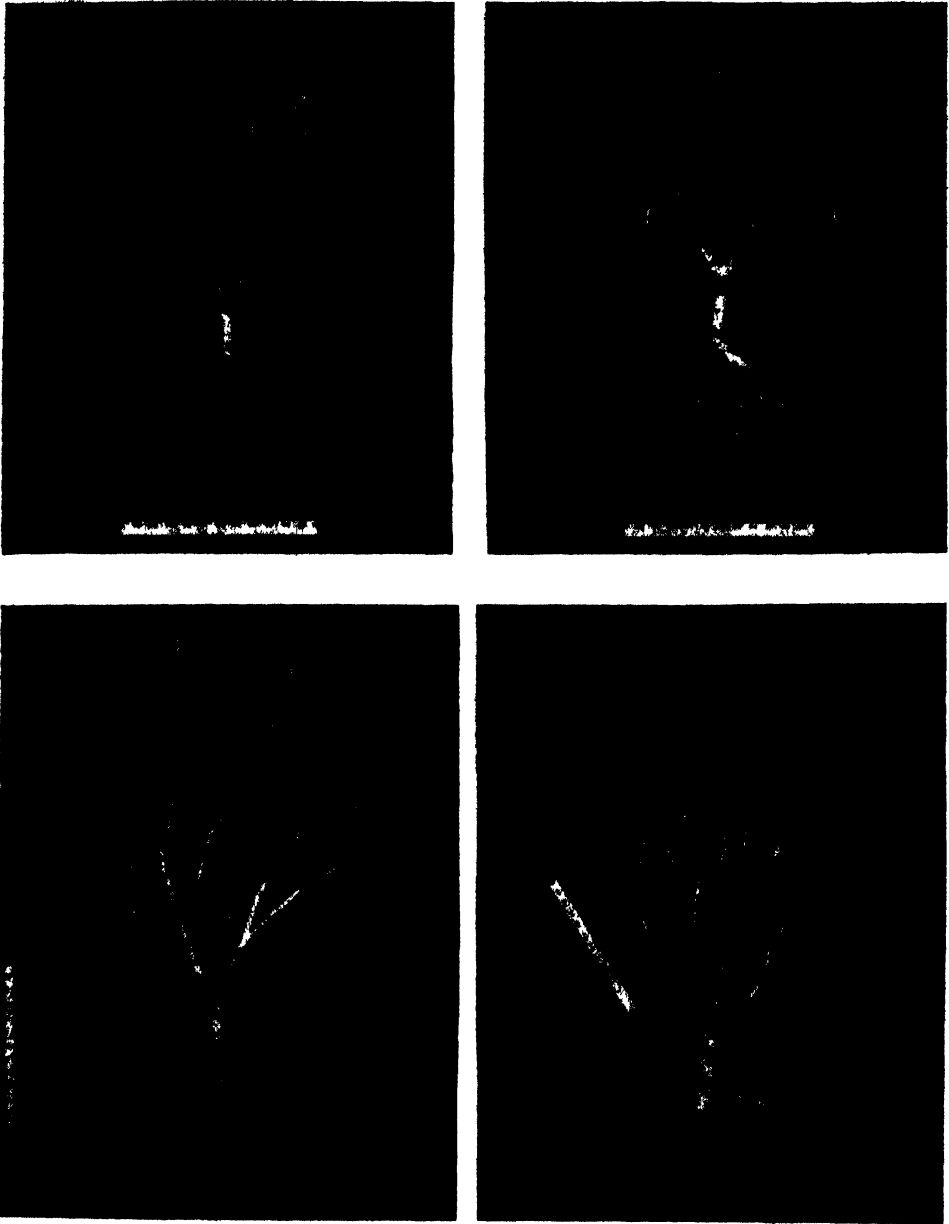
FIG. 1. Appearance of guayule plants as taken from the field (left) and the segments into which defoliated plants were separated (right). Above is a two-year-old irrigated plant showing segments branch roots, root, crown, 1st year stem, and 2nd year stem in upward sequence from the base. Below is a nine-year-old plant and the segments branch roots, root, crown, 1st year stem, 2nd year stem, 3rd and 4th year stem (combined), 5th and 6th year stem (combined), 7th and 8th year stem (combined), and 9th year stem. The rule appearing in each photograph is 12 inches (30.5 cm.) long.

response to exhaustion of available soil moisture or low temperature. Cycles of growth are consequently separated by regions in which the shortened internodes leave a collar of leaf scars similar to the bud scars occurring on deciduous woody plants. The annual increments of growth in length are

·clearly distinguishable on young plants growing in the vicinity of Salinas; but on plants approaching ten years of age the markings on the older parts become obscure, and in regions where irregular climatic patterns of moisture and temperature occur, annual increments could not be located accurately.

Dead twigs and branches and the remnants of the wiry flower peduncles were removed as a separate sample from the older parts, and the stem of the most recent growth increment was separated from its attached leaves and flower peduncles. The terms "bark" and "wood" refer to the groups of tissues obtained by separation at the cambium region. By hammering the segments of stem lightly, the tissues can be separated where the fragile cells of the cambium region have been crushed. .

Following coarse chopping by hand, the various fractions of plant material were dried at 65° C., then ground successively through a Wiley mill and a hammer mill (9). Rubber and resins were usually determined by the method of SPENCE and CALDWELL (11). The term "resins" thus refers to the substances which may be extracted with acetone after the sample has been boiled with dilute acid and thoroughly leached with water. Rubber was extracted with benzene following the removal of the acetone-soluble substances. Other methods for rubber (12, 14) were employed in special cases as indicated in the text. Percentage contents of rubber and resin are expressed as percentage of dry weight.

The plants of tables I to VI were all sampled in April when growth was just beginning and the rubber concentration was at a maximum which would not be exceeded until the following autumn. In connection with total yields from the plants, it should be mentioned that with plant spacings as wide as those involved in these fields, harvesting is not considered economical until more than three years of growth have elapsed. The plants do not fully occupy the field area until this time, and during the earlier years the annual increments of rubber increase with age.

Statistical analyses was by the method of analyses of variance. From the error variance the standard error of difference between means was ascertained, which by reference to the tables of "t," provided an estimate of the difference required for significance (5).

Results and discussion

TWO-YEAR-OLD PLANTS

Table I records the distribution of weight, rubber, and resin among the various segments of root and stem. These plants, taken from the first plantation established in the operations of the Emergency Rubber Project, had grown for two years in the field without any irrigation to supplement the winter rainfall of about 13 inches. , Rubber concentration, expressed as percentage of dry weight, is lowest in the root, higher in the branch roots, and much higher in the crown and branches. The oldest segments of the branches, stems formed during the first year, are higher in rubber content than the second year stems; but the difference is surprisingly small in view

TABLE 1

DISTRIBUTION OF RUBBER, RESIN, AND WEIGHT IN TWO YEAR-OLD GUAYULE

NO IRRIGATION TO SUPPLEMENT WINTER RAINFALL; STRAIN 593; SPACED 36 BY 24 INCHES; SAMPLED APRIL, 1944

PLANT PART		RUBBER IN DRY TISSUE	RESIN IN DRY TISSUE	DRY WEIGHT		WEIGHT OF RUBBER		FRESH WEIGHT PER PLANT
				PER PLANT	FRACTION OF TOTAL†	PER PLANT	FRACTION OF TOTAL†	
		%	%	gm.	%	gm.	%	gm.
Branch roots		8.96	7.66	4.9	3.2	0.44	2.6	12
Root		8.01	6.00	26.3	17.1	2.11	12.6	53
Crown		11.43	6.28	18.3	11.9	2.09	12.5	35
1st year stems*		12.07	6.76	50.3	32.6	6.07	36.3	93
2nd year stems		11.05	8.00	54.3	35.2	6.00	35.9	102
Least sign. difference	5%	0.71	0.51	1.0			1.4	
	1%	0.97	0.69	1.3			1.9	
Total plant		10.34	7.04	154.1	100	16.71	100	295

* Stem segments are numbered according to sequence of appearance during the plants' growth. Thus the primary tissues of the 1st year stems were formed in the first year of growth.

† Relative quantity in the part is expressed as percentage of total in the plant.

of the fact that the oldest parts of the former are two years old, while those of the latter are only one year old. It is interesting to consider that although the first year stem contained a considerable percentage of rubber (table VII) before the growth of the second year stem even started, the two are now not greatly different in rubber content.

Resin concentration is also at a minimum in the root, but with only an insignificantly higher concentration in the crown. The second year stem, formed during the past year, shows distinctly more resin than the older stem.

The data show that two-thirds of the dry weight of the plant is in the branch system above the crown, about one-third distributed between the root and crown, and only a very minor part in the secondary roots. The composition of the branches will, therefore, be the predominant factor in gross composition of the plant as a whole.

Distribution of the total weight of rubber among the parts is roughly parallel to the distribution of dry weight. The contribution of the root in terms of rubber quantity, however, is less than in terms of dry weight, because of its low rubber concentration; the contribution of the branch system is correspondingly greater.

Plants represented by table II are also two years old, but they had been irrigated in July and September of each year. Detailed comparisons attributed to irrigation treatment, are not to be made between these tables I and II, for the plants are from independent experiments on separate plantations, the spacings in the field are different, and two varieties are involved. The nominally distinct strains "593" and "406," however, are practically indistinguishable in growth and rubber content (3), so the sharply contrasted levels of rubber concentration probably reflect principally the contrasting moisture conditions of the plants. It is also probably justifiable to note that although the longer period of summer growth provided by irrigation resulted in a lower rubber concentration throughout the plant, a compensatingly higher dry weight resulted. Consequently, the rubber yield per plant is very similar under the two moisture conditions. Such approximately compensating responses to irrigation have commonly been observed when natural conditions have not been extremely xerophytic (4). [2,3]

Despite the over-all dissimilarity between populations, the relations within the plants of table II are similar to those shown in table I. Rubber concentration is lower in the root than in any other part. Here, however, the branches are significantly higher in rubber concentration than in the crown, and the younger second year stem not only approaches but even equals the first year stem in rubber concentration. Resin concentration is again at a minimum in the root, and the second year stem is richer in resin

[2] HOLMES, RAIFORD L. Analyses of samples from indicator plots taken in February 1944. Unpublished data summarized in the files of the Emergency Rubber Project, Salinas, California. 1944.

[3] TINGEY, D. C. The effect of different frequencies of irrigation on two-year-old guayule. Unpublished report in the files of the Emergency Rubber Project, Salinas, California. March 17, 1945.

TABLE II

DISTRIBUTION OF RUBBER, RESIN, AND WEIGHT IN IRRIGATED TWO-YEAR-OLD GUAYULE

IRRIGATED IN JULY AND SEPTEMBER OF EACH YEAR; STRAIN 406; SPACED 28 BY 24 INCHES IN FIELD; SAMPLED APRIL, 1944

PLANT PART	RUBBER IN DRY TISSUE BARK %	RUBBER IN DRY TISSUE WOOD %	RUBBER IN DRY TISSUE WHOLE %	RESIN IN DRY TISSUE BARK %	RESIN IN DRY TISSUE WOOD %	RESIN IN DRY TISSUE WHOLE %	DRY WEIGHT PER PLANT BARK gm.	DRY WEIGHT PER PLANT WOOD gm.	DRY WEIGHT PER PLANT WHOLE gm.	DRY WEIGHT BARK PERCENTAGE %	DRY WEIGHT FRACTION OF TOTAL %	WEIGHT OF RUBBER PER PLANT BARK gm.	WEIGHT OF RUBBER PER PLANT WOOD gm.	WEIGHT OF RUBBER PER PLANT WHOLE gm.	WEIGHT OF RUBBER FRACTION OF TOTAL %	FRESH WEIGHT PER PLANT gm.	FRESH WEIGHT BARK PERCENTAGE %
Branch roots	....	....	4.91	....	....	6.40	....	....	22.7	....	8.6	....	....	1.12	6.7	48	....
Root	10.01	0.85	3.90	8.40	2.34	4.33	12.8	25.8	38.6	33.2	14.6	1.28	0.22	1.51	9.0	76	43.4
Crown	10.19	2.93	6.12	8.12	2.91	5.22	13.0	16.6	29.6	44.0	11.2	1.32	0.49	1.81	10.8	56	50.2
1st yr. stems	9.72	3.59	7.13	7.90	3.10	5.88	45.2	32.9	78.1	57.9	29.6	4.39	1.18	5.57	33.1	142	62.5
2nd yr. stems	8.95	4.12	7.22	7.91	5.92	7.20	60.5	34.0	94.5	64.0	35.9	5.42	1.40	6.82	40.5	177	68.0
Least sign. 5%	0.43	0.24	0.35	0.64	0.55	0.28	..	..	..	1.3	2.1	..	..	..	2.0	..	1.4
difference 1%	0.58	0.33	0.48	0.89	0.76	0.37	..	..	..	1.9	2.9	..	..	..	2.7	..	1.9
Total plant	....	....	6.39	....	....	6.09	..	..	263.5	..	100	..	..	16.83	100	499	....

than is any other part.   The two stem increments together contain about two-thirds of the total dry weight of the plant, and because of their relatively high rubber concentration, nearly three-fourths of the total rubber.

Separation of the various segments into bark and wood portions reveals partial explanations for the pattern of rubber concentration among the whole segments.   Of particular significance in this connection is the fact that rubber is concentrated primarily in the bark; also of importance is the fact that the proportion of bark increases progressively from the root upward to the most recent stem segment.   Thus, although the root bark is as high in rubber as the bark of any other part, there is proportionally much less of the rubber-rich bark in the root sample.   This, coupled with the very low rubber content of the root wood, produces a relatively low rubber concentration for the root as a whole.   In the matter of the second year stem's attaining a rubber concentration approaching that of the older first year stem, the bark of the younger stem yields a rubber concentration quite close to that of the older stem.   Even this difference is counteracted by the greater proportion of bark in the young stem and to a slight extent by the higher concentration in the wood, so that the resultant concentration of the two stem segments is nearly identical.   Rubber concentration of the bark fraction is at a maximum in the root and the crown, somewhat lower in the first year stem, and still lower in the young second year stem.   The wood on the other hand exhibits an extremely low rubber content in the root, and the concentration rises progressively through the upper parts of the plant.

Resin, as rubber, finds its site of principal concentration in the bark.   It is distributed rather uniformly through the bark of the various segments. A slight gradient upward is suggested in the wood, but the only impressive distinction is in the high resin concentration of the wood in the second year stem.   This contributes to the markedly high resin content of the second year stem as a whole; differences in resin concentration among the other whole segments are largely reflections of varying proportions of bark and wood.

When rubber content is considered in terms of the actual amount in the parts, it is seen that the major part is carried in the bark, as would be indicated from the distribution of weight and rubber concentration.   Depending on the segment, the amount in the bark comprises from 72% to 85% of the total.   Of the total rubber in the plant 79% is carried in the bark tissues. This figure ignores the branch roots, but the undetermined distribution there would not alter the figure greatly, because when included they contain less than 7% of the total rubber observed.

THREE-YEAR-OLD PLANTS

Tables III and IV present data for plants which have grown for three years in the field.   All were irrigated once during the first year but not during the second year.   In the third year the plants of table III continued without irrigation, while those of table IV received a single irrigation in

TABLE III

DISTRIBUTION OF RUBBER, RESIN, AND WEIGHT IN THREE-YEAR-OLD GUAYULE
IRRIGATED IN FIRST YEAR ONLY; STRAIN 130, SPACED 36 BY 24 INCHES; SAMPLED APRIL, 1944

PLANT PART	RUBBER In Dry Tissue — Bark %	Wood %	Whole %	RESIN In Dry Tissue — Bark %	Wood %	Whole %	DRY WEIGHT Per Plant — Bark gm.	Wood gm.	Whole gm.	Bark percentage %	Fraction of total %	WEIGHT OF RUBBER Per Plant — Bark gm.	Wood gm.	Whole gm.	Fraction of total %	FRESH WEIGHT Per Plant gm.	Bark percentage %
Branch roots	----	----	7.62	----	----	7.21	----	43.3	14.0	----	3.5	----	----	1.07	2.5	26.9	----
Root	17.55	2.03	6.80	9.71	3.57	5.46	19.2	24.1	62.5	30.7	15.6	3.37	0.88	4.25	9.9	111.0	37.5
Crown	17.70	5.25	10.47	7.47	3.98	5.46	17.2	47.2	41.3	41.6	10.3	3.05	1.27	4.32	10.1	72.3	46.2
1st yr. stems	16.21	6.27	11.61	8.37	3.81	6.25	54.7	51.4	101.9	53.6	25.4	8.86	2.96	11.82	27.5	175.2	57.2
2nd yr. stems	14.89	7.20	11.99	8.06	3.44	6.31	85.0	14.8	136.4	62.3	34.0	12.68	3.69	16.35	38.1	232.5	65.5
3rd yr. stems	12.84	7.63	11.16	9.94	8.56	9.50	30.6		45.4	67.2	11.3	3.93	1.13	5.06	11.8	84.2	70.0
Least sign. 5%	0.62	0.30	0.51	0.55	0.30	0.46				1.4					3.4		2.1
difference 1%	0.85	0.42	0.71	0.76	0.42	0.62				2.0					4.6		2.8
Total plant	----	----	10.86	----	----	6.46	----	----	401.5	----	100	----	----	42.87	100	702	----

TABLE IV

DISTRIBUTION OF RUBBER, RESIN, AND WEIGHT IN "IRRIGATED" THREE-YEAR-OLD GUAYULE

IRRIGATED IN SEPTEMBER OF THIRD YEAR (1943) AS WELL AS IN FIRST YEAR; STRAIN 130, SPACED 36 BY 24 INCHES; SAMPLED APRIL, 1944

PLANT PART	RUBBER IN DRY TISSUE			RESIN IN DRY TISSUE			DRY WEIGHT					WEIGHT OF RUBBER				FRESH WEIGHT	
	BARK	WOOD	WHOLE	BARK	WOOD	WHOLE	PER PLANT BARK	PER PLANT WOOD	PER PLANT WHOLE	BARK PERCENTAGE	FRACTION OF TOTAL	PER PLANT BARK	PER PLANT WOOD	PER PLANT WHOLE	FRACTION OF TOTAL	PER PLANT	BARK PERCENTAGE
	%	%	%	%	%	%	gm.	gm.	gm.	%	%	gm.	gm.	gm.	%	gm.	%
Branch roots	.....	.....	6.41	.....	.....	6.10	.....	.....	18.8	.....	4.4	.....	0.78	1.21	3.1	33	.....
Root	15.45	1.62	5.68	8.70	2.96	4.64	19.9	48.2	68.1	29.2	16.0	3.08	1.11	3.87	10.0	116	36.5
Crown	16.19	4.13	9.13	6.85	3.50	4.89	19.1	26.9	46.0	41.5	10.8	3.09	2.35	4.20	10.9	81	45.9
1st yr. stems	14.29	5.31	10.20	7.82	3.73	5.94	52.2	44.2	96.4	54.2	22.7	7.46	2.83	9.83	25.5	166	57.9
2nd yr. stems	13.06	5.63	10.18	7.88	3.63	6.24	78.8	50.0	128.8	61.2	30.3	10.29	1.25	13.12	34.0	220	64.8
3rd yr. stems	11.46	5.50	9.43	9.44	7.18	8.67	44.0	22.7	66.7	66.0	15.7	5.04	.....	6.29	16.3	125	69.8
Least sign. 5%	0.78	0.54	0.55	0.45	0.24	0.38	.....	.....	.....	1.5	4.0	.....	.....	.....	4.0	.....	1.7
difference 1%	1.08	0.74	0.74	0.62	0.34	0.51	.....	.....	.....	2.0	5.5	.....	.....	.....	5.5	.....	2.4
Entire plant	.....	.....	9.07	.....	.....	6.14	.....	.....	424.8	.....	100	.....	.....	38.52	100	741	.....

mid-September which brought renewed terminal growth and a crop of flowers.

Distribution through the root and stem segments (table III) is quite similar to that found in the younger plants (tables I and II). The branch roots have a higher rubber concentration than the main root, and the concentration increases from root to crown and from crown to first year stem. Not only does the second year stem have a rubber concentration comparable to the older first year stem; but the third year stem, originating entirely in the last of the three years of growth, has attained a concentration closely approaching that of the stems which have been accumulating rubber for more than two years.

Resin concentration reaches a conspicuous maximum in the most recent stem segment, the third year stem, as it did in the second year stem of the two-year-old plants. The second year stem, which presumably had a distinctively high resin content a year previously, now has the same concentration as the older first year stem. Root and crown show lower resin contents than any other parts. Stems of the above-ground branch system carry about two-thirds of the total dry weight and three-fourths of the total rubber, as they did in the case of the smaller two-year-old plants.

Within the bark portion of the plant, the root and crown show the highest rubber concentration. Above the crown, the concentration decreases with decreasing age of the stem segment. In the wood, the lowest rubber concentration occurs in the root, with an abrupt rise in the crown, and a slight rising gradient in the progressively younger stem portions. In these two tables the data for the bark and wood of the third year stem cannot be strictly compared to the corresponding tissues of the other segments by the indicated statistical standards. Samples of this stem segment were separated in only three of the six replicates. Furthermore, some selection of material was necessary to obtain stems which could practicably be separated into bark and wood; and the analyses were by the photometric, micro-method of TRAUB (12). However, the relative weight of dry material, rubber, and resin in the bark as compared to the wood was quite consistent, so the information from these restricted data is included for comparative purposes. According to these relative weights, the observed values for the whole stem samples were calculated into their bark and wood components to give figures comparable to those from direct analysis of the whole stems.

Resin distribution within the tissues does not show a striking pattern except for the high concentration in the root bark, the high level reached in both the bark and wood of the third year (one-year-old) stem as compared to the older stem segments, and of course the relative richness of the bark as compared to the wood.

Dry weight distribution between bark and wood displays the same relations observed in table II. In the root the bark contributes less than one-third of the dry weight. Proceeding upward in the plant to younger parts the proportion of bark progressively increases until it represents about two-

thirds of the dry weight in the case of the third year stem. The total amount of rubber deposited in the bark of the various segments comprises 71% to 79% of the total for the whole segment. Omitting the branch roots, 76% of the rubber in the plant is contained in the bark.

As a result of irrigation, the plants of table IV show a lower rubber concentration than those of table III. However, little change in the pattern of distribution can be seen. The lower level of rubber content is reflected rather uniformly through all of the parts. Resin, though perhaps at a slightly lower general level, is also very similar in distribution. Dry weight is distinctively different only in the greater weight of the third year stem. This table is offered only to show the effect of distinctive irrigation response on relative distribution within the plant, not as a typical example of irrigation culture to compare with the table III plants which were not irrigated. The terminal growth made possible by the single late irrigation was soon terminated by low temperature; and moisture differentials prevailed only until the rains of late autumn and winter.

NINE-YEAR-OLD PLANTS

Some of the relations shown in the preceding tables had already been observed in earlier samplings of the three-year-old population. It had been particularly striking that the young third year stems yielded a rubber concentration approaching that of the older stems, and that the second year stems were as high in rubber as the first year stems which were a full year older. To further investigate this tendency of young stems to attain, within one or two years, the same rubber concentration as older stems on the same plant, samples were taken from a population of nine-year-old plants (table V). With these plants it would be possible to compare a series of stem segments in which the oldest tissues ranged from one to nine years of age.

The appearance of these plants, and the segments into which they were separated are illustrated (fig. 1). Five samples of plants were separated, with two plants combined for each sample. The separation was similar to that performed on the younger plants, except that the stems were divided into biennial increments between the third and eighth year of annual elongation. Because the markings were occasionally obscure, the stem segments may not have been accurately distinguished in all cases. Nevertheless, the series from the base of the branches to the tips comprises a series of samples with decreasing average age. The planting received no irrigation until the seventh year of growth in the field, and was irrigated each summer thereafter. The relatively longer and heavier segments in the seventh and eighth year increment, which seemed anomalous before the irrigation history could be ascertained, are probably a response to the improved moisture supply.

Because of the irrigation imposed during the last three years it is impossible to draw definite conclusions concerning a tendency for equilibrium in rubber concentration through segments of varying ages. Although a gradient of decreasing rubber concentration from the first year stem to the

TABLE V

DISTRIBUTION OF RUBBER, RESIN, AND WEIGHT IN NINE-YEAR-OLD GUAYULE

NOT IRRIGATED UNTIL SEVENTH YEAR, IRRIGATED EACH YEAR THEREAFTER; STRAIN 406, SPACED 36 BY 24 INCHES IN FIELD; APRIL, 1944

PLANT PART	RUBBER — In dry tissue: BARK %	WOOD %	WHOLE %	RESIN — In dry tissue: BARK %	WOOD %	WHOLE %	DRY WEIGHT — Per plant: BARK gm.	WOOD gm.	WHOLE gm.	BARK PERCENTAGE %	FRACTION OF TOTAL %	WEIGHT OF RUBBER — Per plant: BARK gm.	WOOD gm.	WHOLE gm.	FRACTION OF TOTAL %	FRESH WEIGHT — PER PLANT gm.	BARK PERCENTAGE %
Branch roots			10.15			8.42			57.9		7.6			5.88	5.7	102	
Root	20.66	3.52	8.76	9.70	5.36	6.78	34.8	79.7	114.5	30.4	15.0	7.18	2.81	10.03	9.8	187	36.2
Crown	21.82	7.77	13.27	6.97	4.55	5.30	43.1	66.7	109.8	39.2	14.4	9.40	5.18	14.57	14.2	178	43.3
1st yr. stems	21.66	9.28	15.86	8.87	4.29	6.71	61.7	53.9	115.6	53.4	15.1	13.36	5.00	18.33	17.9	187	57.1
2nd yr. stems	20.46	8.11	15.55	8.76	4.03	6.84	66.0	43.5	109.5	60.3	14.3	13.50	3.53	17.03	16.6	181	63.8
3 & 4 yr. stems	19.39	8.28	15.32	9.10	4.07	7.26	56.0	32.4	88.4	63.4	11.6	10.86	2.68	13.54	13.2	146	66.1
5 & 6 yr. stems	18.28	8.15	14.90	9.87	4.15	7.96	39.8	20.0	59.8	66.6	7.8	7.28	1.63	8.91	8.7	97	69.0
7 & 8 yr. stems	16.21	7.88	13.54	10.43	6.61	9.21	61.9	29.1	91.0	68.0	11.9	10.03	2.29	12.32	12.0	150	69.8
9th yr. stems			9.88			10.68			18.5		2.4			1.83	1.8	33	
Least sign 5%	0.55	0.54	0.45	0.54	0.39	0.50				1.7							1.6
difference 1%	0.75	0.73	0.61	0.75	0.43	0.69				2.3							2.3
Entire plant			13.39			6.97			765.0		100			102.49	100	1261	

sixth year stem is suggested, the amount of change is very slight. A noticeable decrease is seen when the seventh and eighth year segment is reached, but since all the tissues of this segment were developed in the years when irrigation prevailed, it is not known whether an effect of age or of irrigation is reflected. The magnitude of the decrease is relatively small in any case. In the ninth year (one-year-old) stem, however, a greater decrease occurs. and this segment as compared to the preceding one probably does not reflect an irrigation effect alone. Thus it appears that in an old plant a recently developed stem segment will not attain a rubber concentration comparable to the older segments until more than a year has elapsed. Within another year or two, however, it will have a concentration only slightly lower than that of stem segments in which the oldest parts are several years older.

With separation of the segments into tissues, the bark shows a maximum rubber concentration on the crown and the first year stem, with a slightly lower value in the root. From the first year stem upward, rubber concentration in the bark decreases along a gradient with decreasing age of the stem. In the wood fraction, the now familiar low concentration associated with the root occurs again. Through the other segments of wood, rubber is at a rather uniform level, except that a distinct maximum appears in the first year stem.

Resin concentration for the whole segments is at its lowest value in the crown. Above the crown a slight rising gradient is suggested, but marked increases occur only in the two youngest segments. Practically the same pattern occurs in the bark. The wood attains its highest resin content in the youngest stem segment, is at a rather uniform level in the older stems, but seems to rise progressively in the segments below and older than the second year stem.

The proportion of dry weight contained in the bark follows the same pattern as in the younger plants. The root contains the smallest proportion of bark. Upward from the root, a gradient of increasing relative bark weight extends to the youngest stems. This gradient operates to maintain a relatively uniform rubber content for the stem segments as a whole, in spite of the decreasing gradient of rubber in the separate bark and wood fractions.

Distribution of the total dry weight in the plants shows that the root comprises about the same proportion as it did in both the two- and three-year-old plants. Evidently total growth in the branches and roots occurs at approximately the same relative rates. Although the proportion of the total weight contained in the crown was similar between the two- and three-year-old plants, it appears to be higher in the nine year plants. But this is probably due to the arbitrary method of choosing the upper limit of the crown; because of the swollen development of the old crown, presumably engulfing the basal portion of the branches, more of the lower part of the branches may have been included in the crown portion of these older plants than in the younger plants.

Of the total rubber contained in the plants, about 70% is in the stems above the crown. The amount contributed by the bark ranges from 65% to 82% among the several segments. Ignoring the branch roots and the ninth year stem, which carry only a small part of the total, 76% of the rubber in the plant is confined to the bark.

Because of the high concentration of rubber and resins in bark tissues the manipulation in grinding, subsampling for extraction, and extraction frequently presents difficulties. This was particularly evident in the nine-year-old plants because of the high concentrations involved. To see if these difficulties were likely to lead to erroneous results, subsamples of entire stems were taken at the same time that the subsamples were taken for tissue separation. The rubber and resin contents determined directly on the samples of whole stem were then compared to those calculated from the analyses of separate bark and wood fractions. In thirteen such comparisons involving stems of various ages, the mean rubber content of the whole stems analyzed directly was 15.06%, and that obtained from separate analyses of bark and wood was 14.88%. A difference of 0.28% would be required for significance at the 5% level. The resin values were 7.42% when determined directly and 7.58% when calculated from separate analyses of the parts, with a difference of 0.19% required for significance. Thus it seems that, at least at this season, the composition obtained from analysis of separate tissues does not deviate conspicuously from that obtained in the commonly employed samples of whole stems or plants.

DEAD PARTS AND LEAVES

In the preceding tables the figures for the entire plant do not take account of the dead stems and flower peduncles or of the leaves. Therefore, to provide for a more complete description of the plants as a whole, the quantities for these parts are offered (table VI).

In the composite collection recorded as dead parts, dead shoots and the peduncles remaining from former flowers occur in varying proportions, depending on the age and history of the plant. Less than one-tenth of the dead material on the two year unirrigated plants consists of dead vegetative shoots, so the sample of dead portions is almost entirely composed of flower peduncles. On the two-year-old irrigated plants, dead shoots and twigs compose nearly a third of the total dead parts, on the three-year-old plants nearly two-thirds, and on the nine-year-old plants twigs and branches which have died back compose all but a small percentage of the weight recorded. These varying proportions of flower peduncles are largely responsible for the varying rubber and resin contents for the dead tissue sample. Only negligible amounts of rubber (about 0.3%) are to be found in the peduncles; and the resin content (between 2 and 4%), is also lower than that of dead vegetative stems.

With increasing age of plant the relative weight of dead parts evidently increases. Much of the increase is due to death of branches and twigs

resulting from competition within the plant, and perhaps to some extent from cultivation injuries. When the rubber and resin content of the whole plants (tables I–V) is corrected to include the dead parts, the resultant figures represent the values to be obtained for whole defoliated plants as they are usually taken for analysis or commercial milling. The effect of the dead parts is to lower both rubber and resin concentration only slightly in young plants, but to lower rubber more conspicuously and to raise the resin concentration as advance age increases the proportion of dead twigs and branches.

Leaves are not usually included in the weight of plant material as it is

TABLE VI

DEAD PARTS AND LEAVES
(FOR PLANTS OF TABLES I–V)

PLANT PARTS AND CONSTITUENTS	PLANT POPULATION SAMPLED				
	2 YR. I*	2 YR. IRRIG. II	3 YR. III	3 YR. IRRIG. IV	9 YR. V
Dry weight of dead stems					
Grams per plant	13.0	19.0	66.0	73.0	190.0
Per cent of entire defoliated plant	7.8	6.7	14.1	14.7	19.9
Percentage rubber					
Dead stems	0.7	1.1	2.6	2.5	5.4
Defoliated plant less dead stems	10.8	6.4	10.7	9.1	13.4
Defoliated plant plus dead stems	10.5	6.0	9.5	8.1	11.3
Percentage resin					
Dead stems	2.9	4.1	7.0	7.0	13.3
Defoliated plant less dead stems	7.1	6.1	6.1	6.2	7.0
Defoliated plant plus dead stems	6.7	5.9	6.5	6.3	8.4
Leaves, fresh weight					
Grams per plant	93.0	106.0	156.0	178.0	140 0
Percentage of entire leafy plant	30.0	20.0	20.0	22.0	9.0
Leaves, dry weight					
Grams per plant	36.0	42.0	63.0	75.0	52.0
Percentage of entire leafy plant	18.0	13.0	12.0	13.0	5.0

* Number of table in which same plant population is considered.

used for experimental studies of yields or for milling operations. The very low rubber content makes the leaves, at best, a mere dilution factor with a wide seasonal variation; and they are readily removed by a parboiling process employed by the Intercontinental Rubber Company. After immersing the plants in boiling water for a period of five to twenty minutes, the leaves can easily be shaken from the plants. However, to more completely describe the plants, the observed weights of leaves are added (table VI).

A conspicuous feature in the leaf weights appears only in the relatively small proportion of leaves on the old plants. This merely confirms a visible impression. On the tall old plants, crowded in the row, leafy shoots are confined to a horizontal plane at the top of the plant, whereas on the young plants the leafy shoots form a complete hemispherical canopy over the naked lower branches. The leaf weights recorded here are representative only of the early spring period of minimum leaf weight, just as the terminal buds

are beginning a new cycle of growth. As the leafy shoots develop in the spring and summer, the leaves of the last year cycle become senescent and wither, and by midsummer have been almost completely removed by mechanical action of wind and cultivation. In the case of the two- and three-year-old plants considered here, the total weight of leaves doubled by midsummer, and the proportional weight of leaves increased by approximately 50%. Following this maximum, representing almost entirely the leaves on the newly formed shoots, the leaf weight dropped again toward the minimum of the following early spring. The leaf samples recorded showed a mean rubber concentration of 0.3% to 0.5% and a resin content of about 10%. The rubber content is quite typical for leaves, though it may occasionally approach 1%; the resin value seems to represent a late winter maximum in a range which displays values of 6% to 8% in other seasons.

The composition of the dead branches and twigs of the nine-year-old plants offers a pertinent suggestion. Although many of the branches had grown for several years before they died, and would presumably have had fairly high rubber content, the rubber concentration of the dead sample is lower than that of any stem segment of even the two-year-old plants. Associated with this low rubber content is an extremely high resin content. This suggests the possibility that in the dead tissues rubber had been oxidized to substances which are soluble in acetone. Other interpretations are possible, however, and the possibilities were checked in the following manner. Lengths of defoliated stem were oven dried, then suspended from stakes in the field where they were exposed to the weather from October to February. The rubber content during this period dropped from 9.2% to 3.6% while the resin content rose from 7.0% to 12.1%; the differences required for significance are respectively 1.0 and 1.5 at the 1% level. Thus it seems that dead tissues in the field are very quickly subject to changes which transform the rubber into acetone soluble products. Probably sunlight is a major factor in this change, for the deleterious effects of light on rubber in vitro have long been recognized, and dried ground tissue samples held in the laboratory show little or no change over such a short period.[4] Paralleling this group of dead stems, defoliated fresh stems were exposed in the field to follow the changes associated with more natural death. However, in the damp cool weather natural drying proceeded very slowly. During the first two months the stems remained quite fresh in appearance, and after four months exposure they still contained 23% moisture as compared to an initial 40%, and to the 12% which dried stems had acquired in equilibrium with the field atmosphere. No perceptible change had occurred in the rubber content at this time, and resin content had actually decreased slightly.

ONE-YEAR-OLD PLANTS

Although in all the cases considered thus far the root has yielded lower concentration of rubber than the stems, this situation may not extend to very

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[4] HOLMES, RAIFORD L. Report of experiment on storing guayule samples. Unpublished report in files of Emergency Rubber Project, Salinas, California. 1945.

young plants (table VII).   In this case the plants approximate one-year-old
field plants in size and rubber content.   However, they are the product of
a direct field seeding planted in September, 1943.   Low temperature brought
a cessation of growth by December, so when growth resumed in the following
spring the above ground portion of the plant was only a rosette.   Practically
all of the stem tissue and more than nine-tenths of the root tissue recorded
for the February, 1945 harvest is therefore a product of the 1944 growing
season.   The planting had not been irrigated since July, and a final thinning
at that time left a field spacing of 20 by 18 inches.   The plant axis was
separated into the primary root, which was cut at about six or seven inches
below the soil surface, and the top portion of combined crown and stems.

In the case of these young plants the rubber concentration of the root is
not significantly lower than that of the stems.   Within the root, the com-

TABLE VII

DISTRIBUTION OF RUBBER, RESIN, AND WEIGHT IN ONE YEAR-OLD GUAYULE PLANTS.   STRAIN
593; SEEDED DIRECTLY IN THE FIELD SEPTEMBER, 1943; SAMPLED IN FEBRUARY, 1945

PLANT FRACTION	RUBBER IN DRY TISSUE	RESIN IN DRY TISSUE	DRY WEIGHT PER PLANT	RUBBER WEIGHT PER PLANT
	%	%	gm.	gm.
Entire plant	5.89	6.09	82.9	4.88
Stems and crown	6.04	6.50	67.3	4.06
Root	5.25	4.33	15.6	0.82
Root bark	10.86	7.15	6.8	0.74
Root wood	0.92	2.15	8.8	0.08

position of bark and wood is comparable to the two-year-old irrigated plants
(table II).   However, the proportion of bark in these smaller roots is
greater, so the resultant rubber concentration of the whole root is higher
than in the older plants.

A few samples collected from a three-year-old irrigated plot in Indio,
California, provided an opportunity to check certain features of distribution
which had been consistently noted in plants grown under Salinas conditions.
The planting in Indio had been irrigated frequently throughout the spring,
summer, and autumn, which with the high temperatures, provided for a
very vigorous growth from spring until late autumn.   This offered a con-
trast with Salinas conditions, where it seems possible that temperature limits
growth even during the summer growing season.   It also provided an ex-
ample of the vigorous plants obtainable under irrigation in those warm areas
of the southwest which seem promising for guayule culture.

Samples were taken in March, 1945, to see if variously aged stem seg-
ments show closely similar rubber concentrations, as they do in Salinas.   On
three-year-old plants, which were spaced 28 by 24 inches in the field, the
rubber concentrations in the first, second, and third year stem segments were
7.1%, 7.1%, and 7.5%, respectively.   Resin concentrations were respectively

5.2%, 5.9%, and 7.1%. These are not dissimilar from the results with Salinas plants, for rubber concentration does not differ among the segments, and resin concentration is markedly higher in the most recent stem segment.

The data from a few plants spaced more closely in the row, 28 by 12 inches, seem anomalous. Rubber percentages were 8.4, 8.4, and 6.1, respectively, for the first year, second year, and third year stem. Distinction of the third annual increment of growth, however, was not certain, and as soon as defoliated weights were available it seemed more probable that only the most recent part of the third annual increment had been taken in the part considered the third year stem. This portion of the stem displayed several increments of growth, as if cessation and renewal of growth had occurred more than once in a year's period. It is possible that the close spacing resulted in deficiencies of water during the summer in spite of the irrigation, and that some of the stem lengths selected as the third year stem contained only the more recent growth of autumn. This together with ARTSCHWAGER's observation (2) that rubber does not begin to appear in cells until they are perhaps two months old or more, might explain the noticeably lower rubber concentration in the part called third year stem. But, of course, even if this were the case, the rubber concentration of the complete third year segment would probably still be lower than the other two annual segments. The comparable rubber concentrations in these two older segments, despite the difference in average age, agree with the results in other collections. Resin distribution is also on a familiar pattern, with percentages of 6.3 and 6.5 for the first and second year stems, and a distinctly higher value of 7.8% for the most recent third year stem.

LABORATORY DETERMINATION OF RUBBER COMPARED WITH MILL EXTRACTION

The question may arise as to whether laboratory determinations of rubber contents can represent the rubber obtainable in commercial extraction, for the methods employed are entirely different. While the laboratory analysis is by means of prolonged solvent extraction, commercial recovery involves mastication of the plant material under water in a pebble mill, and flotation of the resulting agglomerates of coagulated rubber particles (10). Table VIII is an example of a comparison between the two methods. The mill extraction was performed by the Assay Laboratory of the Emergency Rubber Project, by processes which parallel those of commercial scale extraction. Plants of variety 593, grown for three years without irrigation in a field spacing of 24 by 36 inches, were used. Four replicates, involving 40 or more plants for each type of plant material, are averaged (table VIII).

The data illustrate the fact that solvent extraction of plant tissue, as used to give the recorded rubber contents in this report, gives a reasonable estimate of the amount of pure rubber obtainable by pebble-mill procedures. When the crude rubber of the mill product is corrected for the impurities it contains, the resultant yield of pure rubber is very similar to that determined by the Spence-Caldwell solvent method. The amounts of resin and insoluble

materials admixed with the crude rubber are typical of the product from young plants; older plants usually yield somewhat purer rubber. It will be noticed that the proportional amount of resin in the crude rubber is much less than in the original plant material.

TABLE VIII

RUBBER CONTENT OF GUAYULE PLANTS BY MILL EXTRACTION AS COMPARED TO DIRECT CHEMICAL ANALYSIS OF PLANTS

EXTRACTION METHOD AND YIELD	PLANT MATERIAL	
	ENTIRE PLANT	BRANCHES ALONE
	%	%
Mill extraction		
Crude rubber	17.17	18.03
Composition of crude rubber		
Rubber	67.2	66.7
Resin	22.8	21.9
Insoluble	10.0	11.4
Pure rubber obtained by mill process	11.54	12.02
Chemical analysis of plant		
Rubber	11.97	12.41
Resin	8.70	8.92

CONTINUED RUBBER ACCUMULATION IN OLD CELLS

Comparison between the old plants of table V and the younger plants presents another intriguing question. In the root, crown, and older stems of the nine-year-old plants, the rubber concentration in both bark and wood is higher than in the corresponding parts of the younger plants, despite the fact that in the wood of the old stems, bands of dark discolored, presumably dead tissue, were frequently evident. Even in the two-year-old unirrigated plants of table I, with the highest net rubber content of any of the younger plants, the bark yielded 17.4% rubber in the root and 16.4% in the crown, with 1.7% and 5.9% as the respective values for the wood. All these are distinctly lower than the values for the nine-year-old plants of table V. This increase in rubber concentration as well as in total amount may result from two phenomena. Either rubber continues to accumulate in the original cells subsequent to the year of their formation, or the cells added in later years carry sufficiently high rubber concentrations to raise the resultant concentration of the total. Apparently there is no evidence in the literature to show whether a given group of cells will continue to accumulate rubber for several years after their initiation, or whether their rubber content remains unchanged after the first year and is therefore determined by the conditions of that year. This is a question of both theoretical interest and some practical significance. One question in cultural practices, particularly in regard to irrigation, is whether it would be possible to keep the plant in vigorous growth during the early years in the field to produce a large plant,

and then to bring more intense rubber deposition throughout the plant by withholding water.

The wood tissue, or rather the tissues inside the cambium, would seem to offer appropriate material for answering at least the theoretical question concerning the ability of some cells to accumulate rubber after their first year of existence. The tissues are arranged in order according to their sequence of formation in the plant. It follows then, that if the increase in rubber concentration for the wood cylinder as a whole is due to progressive enrichment by more recent layers of wood with high rubber content, the outer layers of wood should exhibit a much higher rubber concentration than the inner portion. On the other hand, if individual cells continue to accumulate rubber over a period of years, the inner wood and pith, corresponding to the entire wood fraction of the segment when it was younger, should yield a higher rubber concentration than is found in the wood of young plants.

A series of such comparisons is presented (table IX). The wood samples employed were from the first year stem. Thus, in the case of the material from ten-year-old plants, the pith and innermost xylem were nearly ten years old. Plants providing the material were from the same planting as the plants of table V, but since the material was taken in the following fall and winter, another year of growth had been added. The lengths of stem were selected in the field to be unbranched and without dead branches. A sample for separation consisted of one or more such lengths taken from each of three to six plants, and from three to six such samples are averaged for each comparison. In the laboratory the bark was removed and the remaining wood and pith portion cut into lengths of approximately 4 cm., which were further selected to omit any which showed remains of branches or bands of dead tissue.

To answer the question investigated, the wood cylinders could be separated into inner and outer layers at almost any intermediate point on the radius, but to provide somewhat more precise information separation was made between specific growth rings. The exposed transverse sections at the ends were polished, and the circumference of the appropriate annual ring was marked under a magnifier. The xylem outside of this ring was then peeled away with a knife to provide the sample of recently formed wood, and the remaining inner cylinder, in which the pith always appeared to be intact, constituted the sample of the early formed wood. Discernment of annual rings is subject to some uncertainty; the same factors that make the detection of annual increments of stem elongation difficult produce even more confusing patterns in the annular pattern of the xylem. However, when the total age is known it is possible to distinguish the annual rings with reasonable confidence. To give an approximation of the proportions of the wood fractions, the approximate outside radii of the several cylindrical fractions and their relative dry weights as percentages of the weight of the entire wood cylinders are included in the table.

TABLE IX

DISTRIBUTION BETWEEN OLD AND YOUNG PARTS OF WOOD CYLINDER (XYLEM AND PITH) IN GUAYULE STEMS

Source of stems*	Comparison	Portion of cylinder	Annual rings included	Rubber in dry tissue	Resin in dry tissue	Outside radius of fraction	Dry weight as percentage of entire wood cylinder
				%	%	mm.	%
Ten-year-old plants, October	A	Inner	1–3	11.90	4.03	3.1	34
		Outer	4–10	5.13	5.74	5.7	66
		Entire	1–10	7.32	5.21	5.7	100
			L.S.D.†	0.92	0.66		
Ten-year-old plants, February	B	Inner	1–3	11.59	3.03	3.7	36
		Outer	4–10	5.88	3.65	6.7	64
		Entire	1–10	7.90	3.41	6.7	100
			L.S.D.†	0.79	0.72		
	C	Inner	1–4	10.14	3.60	3.5	48
		Outer	5–10	5.60	3.45	5.2	52
		Entire	1–10	7.78	3.52	5.2	100
			L.S.D.†	0.84	0.17		
	D	Inner	1–3	11.55	2.83	3.5	32
		Middle	4–6	5.99	3.62	5.0	30
		Outer	7–10	4.05	3.19	6.6	38
		Entire	1–10	7.13	3.19	6.6	100
			L.S.D.†	0.62	0.36		
Four-year-old plants, February	E	Inner	1–2	7.19	3.58	3.0	48
		Outer	3–4	4.71	3.41	4.3	52
		Entire	1–4	5.89	3.49	4.3	100
			L.S.D.†	0.49	0.25		

* The length of stem employed was that formed during the first year of growth; thus ten-year-old stems were used in comparisons A through D, and four-year-old stems in E.

† Least significant difference at the 5% level.

The first comparison (A) (table IX) refers to wood taken in October from the first year stem of ten-year-old plants. Rubber and resins were determined by the photometric method of TRAUB (12). The inner portion includes the pith and the layers of xylem produced during the first three years of growth. It is therefore composed of the same cells which comprised the entire wood cylinder of the stem segment when the plant was three years old. A few of the segments in the samples, however, were separated between the second and third annual rings. From the rubber values of this comparison it is obvious that increased rubber concentration in the wood as a whole with advanced age does not simply reflect an enrichment by later formed tissues with high rubber content. The wood added in the last seven years has a much lower rubber concentration than does the portion which was present at the end of the first three years. Furthermore, the 11.9% rubber now present in the part comprising the first two or three years of growth, but in which the oldest cells are nearly ten years old, greatly exceeds the concentration to be found when the oldest cells were three years old. The wood of the first year stem (tables I, II) after three years of growth contained only 6.3% rubber. The highest value for any five-plant sample included in this average was 6.8% ; during a year of periodic sampling which covered the fourth year of growth without irrigation, the highest rubber content encountered in any individual sample was 7.3%. In the rubber-rich unirrigated plants of table I, samples of the first year wood taken in October after a third summer of growth to compare with the ten year plants, yielded 5.6% rubber with 7.4% as the maximum for any single sample. Therefore it seems probable that the cells of the inner part of the ten-year-old wood, those cells which were present when the wood was only two or three years old, have gained considerable amounts of rubber in the subsequent seven years.

The procedure for the second comparison (table IX, B) was similar to the first one, but the wood samples were taken four months later and the analyses were by the method of Spence and Caldwell. Again the old inner portion of the wood cylinder is not only much higher in rubber concentration (11.6%) than the layers added subsequently (5.9%), but also shows a higher value than it could reasonably have had when its oldest cells were only three years old. When the separation is made so as to include the fourth annual ring in the inner cylinder (C) the results are practically the same. However, the slightly lower rubber concentration of the inner four annual rings as compared to the inner three annual rings may be a true difference, for the net concentrations of the entire wood cylinder happen to be identical for the two comparisons. This slight reduction perhaps reflects a dilution of the three year cylinder by the fourth year wood with a lower rubber concentration. The remaining six outer annual rings of the latter comparison may also be lower in rubber than the outer seven years of the former one because of the somewhat lower average age.

Somewhat more detailed information concerning rubber distribution in the wood is presented in comparison D. To separate the cylinder into three

portions representing a gradation in average age the first three annual rings including the pith were taken as one sample, the succeeding three annual rings as another, and the most recent four annual rings as the third. Rubber concentration decreases progressively with decreasing age which indicates, if nothing more, that the new tissues added with advancing age of the segment would tend to decrease rather than increase the rubber concentration of the wood cylinder as a whole.

There is evidence in this comparison of a situation which is hinted in the previous ones. It appears that toward the innermost part of the wood cylinder rubber concentration rises more abruptly with increasing average age than it does in the outer parts. This suggests that a very high concentration may occur in the innermost tissues, presumably the pith. Unfortunately no samples of pith alone were taken. Although the pith comprises only a minute part of the wood cylinder of older stems, it is possible that the absence of a pith is one factor in the characteristic low rubber concentrations observed in the wood of roots.

The last comparison (E) is within the wood from the first year stem of four-year-old plants. Periodic samples in the same population from which these were taken showed that the rubber concentration of the wood had increased significantly since the previous winter. Such an increase, it is evident from the comparison within the wood, could not have been due to enrichment by a higher rubber concentration in the recently added wood tissue. The recent outer layers have a distinctly lower rubber concentration than does the inner part.

The rubber content in the wood of the old plants in surprisingly high, in view of Whittlesey's report that guayule wood contains no rubber. However, the differing solvent pairs involved in the Spence-Caldwell method and the method of Traub are seen to give very similar results. To further check the identity of the rubber obtained from the wood, the rubber films obtained and weighed in the Spence-Caldwell procedure were submitted to analysis by a gravimetric rubber bromide method (14) immediately after weighing. Eighteen such samples, including those obtained from both old and young wood samples, showed an average purity of 101.5%. Whittlesey's material may have undergone oxidation or offered some of the other difficulties which SPENCE and CALDWELL (11) indicate for the earlier procedures in analyzing plant material. It is possible that the wood tissues contain a less effective supply of natural antioxidants than does whole stem tissue. Stored samples of ground wood tissue seem to decrease in rubber content much more noticeably than bark or composite stem tissue. The rubber films obtained from the benzene extract of the wood samples of table IX did not show any discoloration, and gave the visible appearance of pure rubber.

The very high rubber concentrations in the old inner wood might be interpreted as merely a reflection of disappearance of other constituents in the wood. Although it would be difficult to rule out this possibility completely without following a population over several years, indirect evidence

urges that the results could not be attributed to a decrease in the reference dry weight. A drastic loss of material would be required to cause the observed increase in rubber concentration. Yet ARTSCHWAGER's anatomical observations (1) apparently did not reveal any disintegration of the inner xylem in old plants. Furthermore, if dry material had disappeared from the old xylem it would be reflected either in a reduced ratio of dry weight to cross sectional area or in a compression of the inner cylinder. But calculation from the radii and relative dry weights recorded in table IX indicates that the dry weight/area ratio of the inner wood cylinder is not lower than that of the younger outer wood. Nor is there any evidence of compression; samples of three-year-old wood cylinders from the most vigorous branches of a three-year-old planting showed a mean diameter of 7 mm., similar to that of the inner three years in the old stems of table IX. As a further suggestion, the inner wood of comparison C (table IX) showed carbohydrate reserves at three-fourths the concentration found in the outer wood, and at about the same concentration as normally found in the wood cylinder of three-year-old plants at that season. This indicates that the reference dry weight had not been altered significantly by changes in reserve carbohydrates; and, without any sign that reserves were exhausted, it seems unlikely that extensive dissolution of more permanent xylem constituents had occurred.

Summary

Guayule plants of various ages and cultural histories are separated into several portions to display the distribution of rubber and resins in terms of both concentration and weight.

As the plant is normally harvested for experimental or commercial rubber yields, about two-thirds of the defoliated dry weight is in the branch system above ground, with only one-third in the impressively enlarged crown and root portion. A somewhat larger proportion of the total rubber is contained in the branches, for they usually carry a higher concentration of rubber than does the combined root and crown.

The tissues of the bark are the principal site of rubber deposition. Contrast between the concentration in the bark and that in the remaining wood cylinder is most striking in the root where the concentration in the bark may be eleven times that in the wood. In the branches, however, the concentration in the bark is commonly only two or three times that of the wood; for the plant as a whole the bark has approximately three times the rubber concentration of the wood. In terms of amounts of rubber the bark is even more important, because the weight of bark tissue is somewhat greater than the wood. Between three-fourths and four-fifths of the total rubber in a plant is carried in the bark.

Because rubber is more concentrated in the bark the proportion of bark in the various segments of stem and root is a factor in their resultant rubber

contents.   The proportion of bark is at a minimum in the primary root; consequently, this part shows a lower over-all rubber concentration than the other parts.   From the root upward the proportion of bark increases in the progressively younger parts with smaller diameters.   When the branch system is separated into the successive stem lengths corresponding to successive annual increments of elongation, there is a strong tendency for the uniform rubber concentrations through stem segments of varying average ages.   By the time the oldest tissues in a stem segment are one year old the segment will frequently show as high a rubber concentration as older segments on the same plant.   When another year or two have elapsed the segment will be as high in rubber as earlier formed segments which are several years older.   The greater proportion of rubber-rich bark in the younger stems is an important factor in this apparant equilibrium; in the bark itself there is a noticeable but still relatively slight gradient of decreasing rubber concentration from the oldest stem to the youngest.   Within the wood of plants two or three years old there may be a very slight gradient of increasing rubber from the older stems to the younger ones, but in older plants the gradient disappears or may be partially reversed.

Resins or acetone soluble constituents are also concentrated in the bark. Stems which are no more than one year old show conspicuously higher resin concentrations than do older stems.

Leaves and flower peduncles yield only insignificant amounts of rubber. Dead shoots and branches are relatively low in rubber as compared to live stems.   Evidence is offered to suggest that when a branch dies a considerable portion of its contained rubber is converted to acetone soluble substances within a few months of field exposure.

Evidence obtained by separation of the wood cylinder into annular parts and analyses of the variously aged components indicates that the cells of the inner xylem and pith continue to accumulate rubber for several years after they are formed.   The increased rubber concentration occurring as the wood cylinder becomes older was found to be due to continued accumulation in the old inner tissues rather than to high rubber contents in the xylem added in later years.   The inner annual rings of the xylem were much higher in rubber concentration than the outer ones, and higher than the corresponding annual rings of young plants.

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STUDIES IN THE METABOLISM OF CRASSULACEAN PLANTS: THE DIURNAL VARIATION IN ORGANIC ACID AND STARCH CONTENT OF *BRYOPHYLLUM CALYCINUM*

GEORGE W. PUCHER, CHARLES S. LEAVENWORTH, WANDA D. GINTER, AND HUBERT BRADFORD VICKERY

(WITH EIGHT FIGURES)

Crassulacean plants in general undergo diurnal variation in the acidity of the leaf tissue. They share this behavior in greater or less degree with plants of a number of other families, but crassulacean plants are the classical material for demonstration of the phenomenon; *Bryophyllum calycinum,* in particular, has been repeatedly investigated. Most studies have dealt with the titratable acidity of extracts of the leaves and with attempts to account for the chemical interconversions that take place in terms of reactions in which carbohydrates share. BENNET-CLARK (1) reviewed the earlier literature in 1933 and pointed out that the physico-chemical properties of polybasic organic acids must be taken into consideration in the interpretation of the results of titration. He agreed with previous workers, however, who had claimed that malic acid is the component which increases during the night and decreases during illumination of the leaves.

As a general working hypothesis to account for the observed behavior, BENNET-CLARK suggested that polysaccharides are converted to monosaccharides (sedoheptose, a 7-carbon sugar, in the case of *Sedum praealtum*) which, in turn, are oxidized to malic acid during the period when the leaves are darkened. The reverse reaction occurs during illumination. No evidence was obtained regarding the by-product of the conversion of monosaccharide to malic acid (respectively, compounds containing 6 and 4 carbon atoms) which was assumed to be a compound that contained either 1 or 2 carbon atoms (3 in the case of sedoheptose). The observations suggested, however, that carbon dioxide is not produced, and he expressed the view that, whatever the nature of the by-product, it was probably ultimately converted back into the polysaccharide. Accordingly, in its simplest form, BENNET-CLARK's speculation on the mechanisms that underlie crassulacean metabolism involves an equilibrium between malic acid and a polysaccharide; this equilibrium is upset in one direction or the other according to the conditions of illumination of the leaves.

The most comprehensive investigations of crassulacean metabolism that have been made since the appearance of BENNET-CLARK's review are those of WOLF (14, 15, 16, 17, 18). This investigator studied organic acid fractions isolated by extraction with ether and likewise observed a reciprocal relationship between the quantities of organic acids and carbohydrates, especially starch. He confirmed GUTHRIE's (5) observation that citric acid

undergoes fluctuations parallel with those of malic acid and obtained evidence for the presence of an unknown optically active organic acid which also shares to some extent in the changes.

The identification in this laboratory of isocitric acid as one of the major organic acid components of *Bryophyllum calycinum* leaves (**9, 12**), and also independently at the same time by Nordal[1] (**8**) in two other species of Crassulaceae, has solved the problem of the nature of the so-called crassulacean malic acid and throws light upon WOLF's statements regarding the unknown optically active organic acid of Bryophyllum leaves. Moreover, the development in recent years of analytical techniques for the common plant organic acids as well as the advances that have been made in the theoretical approach to the understanding of carbohydrate metabolism in living tissues suggested that a reinvestigation of one of the oldest problems in plant biochemistry might be rewarding. A report upon the changes in the chemical composition of *Bryophyllum calycinum* over a period of 24 hours of alternate light and darkness is accordingly given in the present paper.

Preparation of samples

A group of 40 young plants that had been transplanted into sand in individual crocks on October 24, 1939 was treated with a complete culture solution containing nitrate as the source of nitrogen [see (**11**) for details of composition] for 126 days when four closely similar large plants and four similar smaller plants were selected for the experiment. On February 27, 1940, a bright sunny day which became cloudy in the middle of the afternoon, a single large and a single small plant were harvested, respectively, at 6:10 A.M. (standard time), 12:10 P.M., 4:10 P.M., and at 6:10 A.M. the following morning. The 6 to 9 fleshy simple leaves in the lowest position on the stem (hereinafter referred to as basal leaves) were collected separately, the petioles being left attached to the stem. The leaflets from the upper compound leaves formed a second and larger sample from each plant. After removal of the basal leaves and leaflets, the stem with the attached petioles was cut at the level of the sand. For each point of time, there were thus two samples of leaves and one of stem and petiole tissue for each separate plant, or 24 in all. The samples were dried and prepared for analysis as previously described (**10**).

Table I shows the numbers of basal leaves and of leaflets, the fresh weights and organic solids, the length of the stem and the organic solids of combined stem and petioles both for the four larger plants and the four smaller plants. In addition, total organic acids and starch of the leaves of the larger plants are included, the data being calculated in terms of grams per single plant. It is obvious that there was appreciable variation in size from plant to plant and that, unless a large number of plants were taken for each sample, clear conclusions could not be drawn regarding

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[1] Because of war conditions, information concerning NORDAL's observation made in 1942 was not received in this country until 1946.

changes in the chemical composition. Accordingly, it was assumed that, notwithstanding moderate differences in size of the individual plants, the *concentration* of the several analytical components would vary with time in a manner that reflected the direction and magnitude of the chemical changes that occur. Analytical data were therefore computed in terms of

TABLE I

FUNDAMENTAL DATA ON SIZE AND WEIGHTS OF *Bryophyllum calycinum* PLANTS
TAKEN FOR ANALYSIS AT INTERVALS OVER A PERIOD OF 24 HOURS.
FIGURES NOT OTHERWISE DESIGNATED ARE GRAMS
PER SINGLE PLANT

TIME OF COLLECTION		6:10 A.M.	12:10 P.M.	4:10 P.M.	6:10 A.M.
ELAPSED TIME (HOURS)		0	6	10	24
Larger plants					
Number of leaves	Compound leaves	20	18	20	18
	Leaflets	96	74	82	78
	Basal leaves	8	6	8	9
Fresh weight	Leaflets	578.4	547.7	512.0	404.7
	Basal leaves	221.5	154.1	190.7	246.0
	Total	799.9	701.8	702.7	650.7
Organic solids	Leaflets	38.5	35.2	31.1	28.0
	Basal leaves	13.7	8.6	11.8	13.2
	Total	52.2	43.8	42.9	41.2
Total organic acids	Leaflets	16.1	11.7	9.1	11.3
	Basal leaves	4.9	2.5	3.2	4.8
	Total	21.0	14.2	12.3	16.1
Organic acids as percentage of organic solids	Whole plant	40.2	32.4	28.7	39.1
Starch	Leaflets	2.0	4.4	4.7	0.9
	Basal leaves	1.0		1.3	0.8
	Total	3.0	> 4.4	6.0	1.7
Length of stem (cm.)		61.5	57.0	62.0	60.0
Fresh weight	Stem and petioles	154.5	132.4	142.5	139.5
Organic solids	Stem and petioles	14.0	11.0	12.5	11.4
Organic acids	Stem and petioles	1.51	1.24	1.21	1.26
Smaller plants					
Number of leaves	Compound leaves	16	14	17	16
	Leaflets	62	43	62	59
	Basal leaves	6	8	7	7
Fresh weight	Leaflets	302.0	208.0	355.2	255.6
	Basal leaves	190.0	124.4	170.9	109.0
	Total	492.0	332.4	526.1	364.6
Length of stem (cm.)		50.0	47.0	54.0	54.0
Fresh weight	Stem and petioles	114.4	87.4	117.1	107.9

1 kilogram of fresh weight of each of the three kinds of tissue examined. This implies the assumption that a kilogram of leaflet tissue collected from Bryophyllum plants in the early morning is, within reasonable limits, the equivalent of a kilogram of the same tissue collected later in the day. Because of biological variation, there can be no certainty that the successive lots of leaflets were identical in composition at the time the first sample was

collected; nevertheless, this is tacitly assumed to have been the case when the composition of such samples is compared and quantitative conclusions are drawn.

The present method of collection of samples was chosen so that the conclusions would be valid with respect to the concentration of components in the tissues of a single whole plant rather than with respect to leaves of a certain arbitrary age or size collected at random from different plants. The separate collection of the basal leaves permitted comparison of the behavior of old with that of younger tissue.

Results

The analytical data are presented as a function of time in the figures. Complete data for the leaflets of the larger plants are shown. The behavior of the smaller plants was essentially a duplicate of that of the larger plants with respect to the directions and even the slopes of many of the curves. In order to avoid unnecessary complication of the diagrams, only part of the data for the smaller plants is therefore presented. The behavior of the basal leaves also in general closely resembled that of the leaflets, but many of the changes were less extensive.

Observations on the leaflets of the larger plants (marked L) are plotted as open circles connected by full lines, those on the basal leaves (marked BL) as open circles connected by broken lines. Data for the smaller plants are plotted as filled circles connected by full lines (marked Ls) for the leaflets and broken lines (marked BLs) for the basal leaves.

CHANGES IN ACIDITY

The top diagram of figure 1 shows the pH of extracts of the dried leaf tissue. Independent experiments showed that determinations of pH made on juice expressed from fresh tissue were identical with those made on extracts from the same sample after it had been dried and, furthermore, that the pH of expressed juice is stable for at least 24 hours and is essentially unchanged even after the juice has been boiled. Any differences observed were at most a few units in the second place of decimals of pH and could be neglected. This result is to be anticipated because of the strong buffering action of isocitric and malic acids in the range of these observations.

The basal leaves were initially at a somewhat higher pH than the leaflets in both the large and the small plants but, in all samples, pH rose steadily during the day and fell during the night. There was a small difference in the magnitude of the change and in the slope of the curves for the basal leaves in the two sizes of plants but the curves for the two sets of leaflets were so nearly identical that only that for the large plants is shown. The magnitude of the change in the leaflets was of the order of 0.75 of a pH unit; thus the actual concentration of hydrogen ions in the plants in the early morning was about 5 times as great as that present in the afternoon. However, this does not mean that there was 5 times as much *acid*, although it indicates a substantial change.

Alkalinity of ash, shown in the middle diagram of figure 1, is expressed in milliequivalents and represents a titration of the inorganic carbonate and alkaline oxide present after all organic matter had been burned away. It is a quantity which would not be affected by changes in the organic acids unless these changes led to transport of inorganic salts into or out of the leaves. In the present case, there was little evidence of such an effect save possibly in the observation at 24 hours for the basal leaves.

ORGANIC SOLIDS

In attempting to interpret the analytical data of the present experiment, it must be held in mind that each point of observation represents the composition of a separate plant. That the plants in each set were not exactly the same in size or weight, in spite of careful selection based on a visual appraisal of their appearance, is evident from the data in table I. Nevertheless, the plants were of the same age, had been grown under as nearly identical conditions as possible and were all members of the same clon. Irregularities less than about 10% that follow no consistent pattern in the curves are accordingly assumed to arise from such factors as biological variation or differences in hydration or both. Changes in the curves that do follow a consistent pattern and which are of a magnitude greater than 10% are assumed to have arisen from actual changes in composition brought about by the conditions of illumination. All statements must therefore be regarded as being subject to these limitations.

The curve for the organic solids of the leaves (fig. 1) furnishes a measure of the limits within which the four plants duplicate each other. The net accretion of organic solids from photosynthesis during the period of illumination was manifestly too small to be demonstrated and was certainly far less than 10% so that change from this cause can be neglected. Loss of organic solids from respiration during the night also fails to show in the curves. The four values for organic solids of the leaflets of the large plants were 66.6, 64.2, 60.8, and 69.2 gm. per kilogram. The mean is 65.2 ± 3.6, the deviation being ± 5.5% of the mean. Thus, the organic solids were constant well within the arbitrary limitation set. The leaflets of the smaller plants and the basal leaves of both sets were even less variable. Accordingly, the actual net changes in over-all organic composition as the result of photosynthesis, respiration, or other chemical change, or in degree of hydration of the tissue were of an order no greater than about 5%.

The curve for concentration of organic solids in the leaflets of the smaller plants lies above that for the larger plants: the smaller plants evidently were somewhat less highly hydrated. The curves for the basal leaves of the two sets of plants are not shown since they were nearly identical with that for the leaflets of the larger plants. In general, then, it is evident that the composition of the leaves of the two sets was sufficiently constant to justify the assumptions made.

The curves for organic solids likewise furnish evidence of constancy of

the hydration since the water content of the samples is the difference between one kilogram and the sum of the organic solids and the ash. The curve for the ash content of the leaflets at the bottom of the figure is very nearly a horizontal straight line. Clearly, the variation in the hydration was within the limits adopted.

NITROGENOUS COMPONENTS

No detailed examination of the nitrogenous components of the samples was attempted but the total nitrogen, protein nitrogen, and soluble nitrogen are shown in figure 2. Total nitrogen of the leaflets of the large plants dropped slightly during the day and the curve resembles that for organic solids in figure 1. The drop might therefore have been a result of a slight increase in hydration rather than an indication of the presence of a smaller actual concentration of nitrogen in the leaflets of the plant collected in the afternoon. Total nitrogen of the basal leaves also dropped but there was no recovery during the following night. The deviation was 4.1% of the mean value for the leaflets of the larger plants and 6.3% for the basal leaves.

The total nitrogen of the leaflets of the smaller plants is also shown. Comparison of these curves with those for organic solids (fig. 1) shows that the variations in nitrogen and in organic solids ran parallel with each other, a further indication that the small irregularities arise chiefly from moderate differences in hydration.

The protein nitrogen of the leaflets underwent a change that was, however, much too large to be attributed to such an effect. During the morning and early afternoon, there was a decrease that amounted to about one-third of the protein present in the early morning, but restoration occurred during the night. The curve for soluble nitrogen showed a rise during the day and a fall at night that was symmetrical both in position and magnitude with the change in protein. The increase in the soluble nitrogen amounted to about 70% of the early morning value. Similar changes were observed in the leaflets of the smaller plants (not plotted). The behavior is consistent with the view that, in the younger leaves, a substantial part of the protein underwent conversion to soluble products[2] during the day but was re-synthesized during the night. No similar behavior was observed in the basal leaves of the larger plants although there was a detectable rise in the soluble nitrogen and fall in the protein nitrogen of the basal leaves of the smaller plants. That the change in the quantity of soluble nitrogen, and, accordingly, also of the protein, was not a result of the change in pH of the tissues can be inferred from these observations on the basal leaves. Furthermore, separate experiments in which the nitrogen extracted was determined after adjustment of the pH of a typical sample of dry leaf to various points within the range of the present experiment likewise showed no effect of pH on the solubility.

[2] That is, to products that are soluble when the dried tissue is exhaustively extracted with hot 70% alcohol and subsequently with hot water in preparation for the determination of protein nitrogen in the residue.

WOLF (**14**), who studied the nitrogen metabolism of crassulacean plants in 1931, did not encounter significant changes in the protein nitrogen of
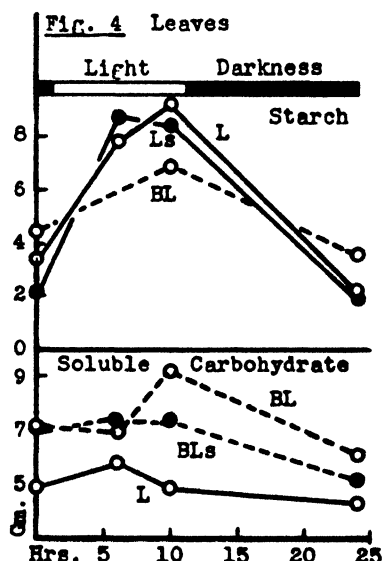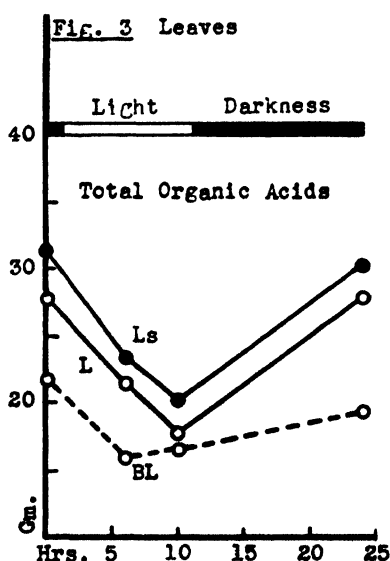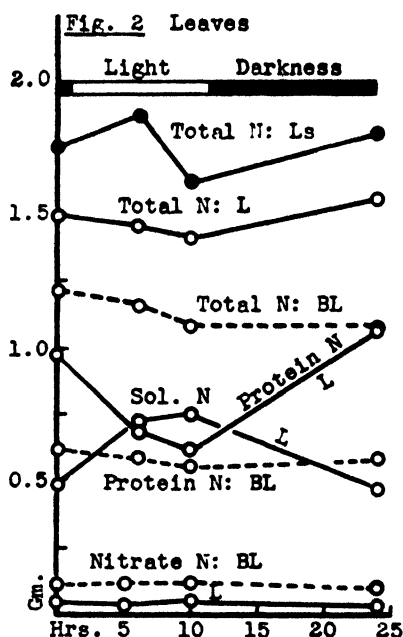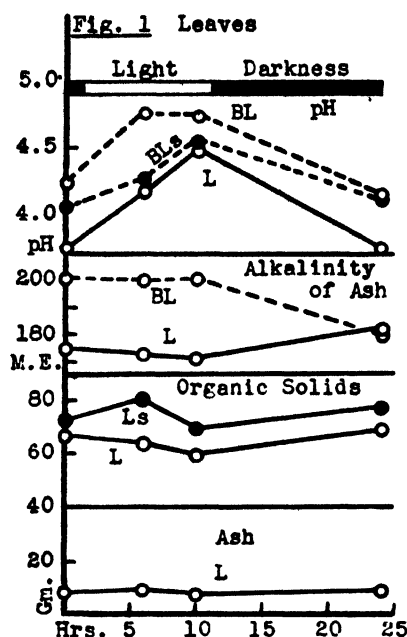
FIGS. 1 to 4. Composition of leaves of *Bryophyllum calycinum* as influenced by light and darkness over a period of 24 hours. Data expressed in terms of 1 kilogram of fresh weight of tissue. The symbol L refers to leaflets of large plants, Ls to leaflets of small plants, BL to basal leaves of large plants, and BLs to basal leaves of small plants.

the leaves. The fact that the basal leaves did not exhibit such changes in the present experiment suggests that the material he studied may have been

too old for easily detectable alterations in the concentration of the protein to occur. He described his material as "*mittelalte Blätter.*"

TOTAL ORGANIC ACIDS

Figure 3 shows the changes in total organic acids, the data from both large and small plants being given. The leaflets lost about one-third of the quantity of acid present at daybreak but this had been completely restored by the following morning. The change in the basal leaves was smaller; the concentration of acids in these was lower to begin with and the loss was only from one-fifth to one-quarter of the amount present in the early morning. Furthermore, for reasons that are not readily apparent, restoration during the night was less complete. Nevertheless, the classical phenomenon of diurnal variation in acidity is clearly evident in the leaves of both sets of plants. The behavior of the basal leaves is similar to that noted by WOLF for old leaves of the same species.

CARBOHYDRATES

Before going into the details of the changes in acid composition of these leaves, the data for starch and soluble carbohydrates (fig. 4) should be considered. Starch underwent a change in concentration in the leaflets reciprocal to that of the total organic acids reaching its maximum late in the afternoon and diminishing during the night. The magnitude of the change was less than that of the change in the acids; the concentration of starch (calculated as glucose) increased by about 6 gm. during the day, the parallel loss in organic acids being nearly 11 gm. During the night, the concentration of starch diminished by about 6.7 gm. while that of acid increased by about 10 gm. In the basal leaves, the loss of acid during the day was about 5.5 gm. and the gain of starch about 2.4 gm. as the average of the two sets of plants, but the gain in acids during the night was only about 2 gm. while the loss in starch was 3.7 gm. Clearly, the figures for loss and gain are in no case equal but they are nevertheless similar to the extent that a large change in the concentration of the acid is associated with a large change in starch.

It must be remembered, however, that the quantities mentioned are concentrations. Reference to table I, which gives quantities per plant, shows that the number of grams of starch laid down in the leaves during the day was far less than the quantity of organic acids that disappeared. On the other hand, the loss of starch during the night and the gain of organic acids were nearly equal. Thus, although it is possible that the acids synthesized during the night may have originated from the metabolism of starch, the reverse of this reaction in daylight could account for only a part of the acids that disappeared and the fate of the balance is by no means clear from the present evidence.

Total soluble carbohydrates (lower part of fig. 4), unlike starch, showed no clearly evident reciprocal relationship with acid content. Although

there was an increase during the day and a decrease at night, the quantities involved were small and the behavior of the large plants differed in detail from that of the small plants. Whether or not the small increases during the day are the result of photosynthesis cannot be demonstrated since there was no significant change in the organic solids. The small decreases at night were associated with a large decrease in starch; thus if starch was converted into a soluble carbohydrate (glucose) as its primary decomposition product, this substance did not accumulate. The velocity of the reactions whereby soluble carbohydrates were transformed into other substances must therefore have exceeded the velocity of the decomposition of the starch.

INDIVIDUAL ORGANIC ACIDS

Details of the changes in concentration of the organic acids are shown in figure 5. The outstanding change occurred with malic acid; this dropped from about 16.5 gm. per kilogram of fresh tissue at daybreak to about 6.7 gm. at 4 in the afternoon but had been restored to about 15.5 gm. by the following morning. The curve for the leaflets of the larger plants only is plotted; that for the smaller plants was identical within a few tenths of a gram at all points.

Diurnal variation in acid content of crassulacean plants has from the time of MAYER been held to arise from changes in the content of malic acid but the evidence has, for the most part, consisted in determinations of the titratable acid as a measure of the magnitude together with the isolation of malic acid from enriched specimens as proof of the nature of the dominant acid.[3] The present data rest upon determinations of malic acid by a specific method and are not open to doubt regarding the identity of the acid that underwent change in concentration. KREBS and EGGLESTON (7) have also demonstrated diurnal variation of malic, citric, and isocitric acids in Bryophyllum leaves by specific analytical methods, and found that alterations in malic acid accounted for 83% of the total change. However, their observation was based on a single brief experiment carried out in the course of the development of an enzymatic method to determine isocitric acid.

Isocitric acid increased in concentration in the leaflets of the larger plants by about 1.5 gm. during the first 6 hours, diminished slightly during the afternoon and remained constant during the night. In the smaller plants, the level of isocitric acid was higher at the start and remained constant during the morning, dropped moderately during the afternoon and remained essentially constant during the night. Isocitric acid in the younger leaf tissue therefore played a subordinate part in the changes in acidity; no clear pattern of change is manifested.

In the older basal leaves, malic acid underwent alterations that were similar to but less extensive than those in the leaflets. Only one set of data is plotted, the differences between the two sets being minor; both showed a substantial loss of malic acid during the day with recovery during the

[3] See, for example, WOLF (14) who isolated and identified malic acid as its silver salt.

night. Isocitric acid was somewhat higher in the basal leaves of the larger plants than in the leaflets at the start but diminished after 6 hours to the same point and thereafter remained nearly constant. The curve is not plotted because throughout most of its course it is indistinguishable from that for the leaflets.

Although citric acid makes up only a minor part of the total acids of Bryophyllum leaves, it shares in the progressive metabolic changes in acidity to a small extent. The data are shown in the lower part of figure 5. In the leaflets, citric acid dropped in concentration during the day by about 0.5 gm. or, roughly, one-fifth of the quantity present at dawn, and increased during the following night to a level appreciably higher than that initially
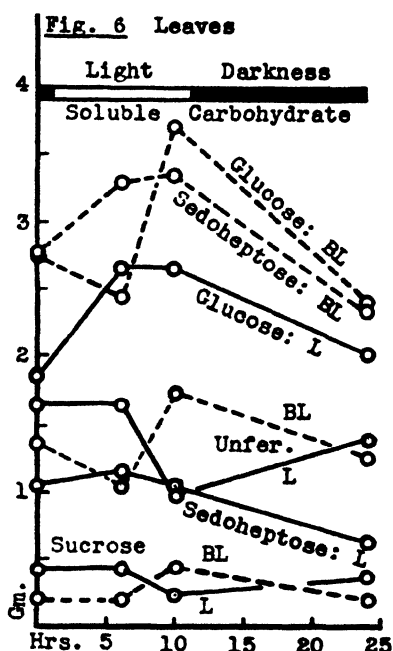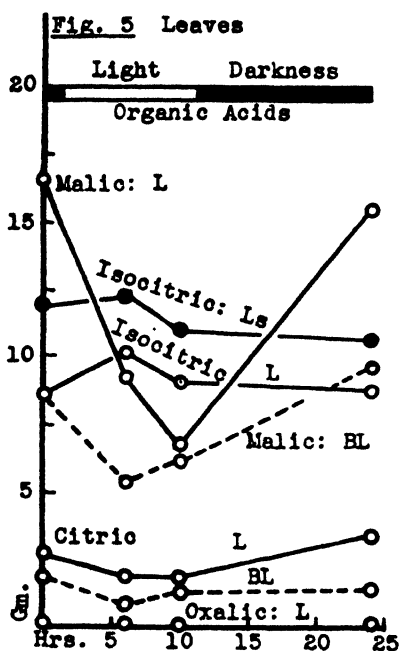
FIGS. 5 and 6. Composition of leaves of *Bryophyllum calycinum* as influenced by light and darkness over a period of 24 hours. Data expressed in terms of 1 kilogram of fresh weight of tissue. The symbols have the same significance as in figures 1 to 4.

present. The leaflets of the smaller plants behaved in the same way. In the basal leaves, the changes were smaller and were probably scarcely significant although a pattern of change similar to that in the leaflets was evident.

That citric acid, like malic acid, normally undergoes diurnal variation in Bryophyllum leaves was first demonstrated by GUTHRIE (5) although the relative importance of the change in the concentration of citric acid in the present material seems to have been rather less than it was in the leaves studied by that investigator. However, the observation has been confirmed by WOLF (17) as well as by BORGSTRÖM (2) and by KREBS and EGGLESTON (7), and the present data furnish further evidence. Nevertheless, the relative order of magnitude of the diurnal variation of citric acid seems always to be much smaller than that of malic acid.

Oxalic acid makes up a minute proportion of the total organic acids of Bryophyllum leaves and the curve for the leaflets (fig. 5) is indistinguishable from the base line. Although there appear to have been slight changes in the concentration and these followed the fall and rise in malic and citric acids, the quantities involved were far too small to suggest that this substance undergoes diurnal variation in concentration. A similar statement is true for oxalic acid in the basal leaves.

SOLUBLE CARBOHYDRATE

Details of the changes in soluble carbohydrates are shown in figure 6 which is plotted on a scale five times that of figure 4. The data do not follow a clear-cut pattern although glucose rose slightly in the leaflets during the day and fell at night. Sedoheptose scarcely changed in concentration in the leaflets during the day but fell at night. In the basal leaves, glucose fell during the first 6 hours, then rose rather sharply during the afternoon and fell during the night. Sedoheptose, however, rose significantly during the day and fell during the night. The data for sucrose show that only minor variation of the small quantity present occurred.

The behavior of soluble carbohydrates in the smaller plants during the day was not exactly like that of the larger plants. None of the data have been plotted in order to avoid unnecessary complexity of the figure. On the whole, the only point on which most of the results agree is that there was a fall during the night.

Glucose is doubtless the monosaccharide in immediate equilibrium with starch but the velocities of the reactions in which this sugar was involved were such that only moderate alterations in the quantity present occurred notwithstanding the large changes in the concentration of starch. The metabolic relationships of the other soluble carbohydrates are by no means clear but, in view of the probable reactivity of these substances, irregularities of the type observed in the curves are all that could be expected. Truly significant results could be secured only if analyses were made of samples that represented large numbers of plants. Nevertheless, it may be noted that sedoheptose in the basal leaves behaved in essentially the manner observed by BENNET-CLARK.

STEM TISSUE

Analytical data for the stem tissue are included to illustrate the contrast in behavior with that of the leaves. Figure 7 shows that the pH underwent diurnal variation but the initial point at pH 4.7 is appreciably less acid than the minimal acidity attained by the leaves in the afternoon. Not only is stem tissue considerably less strongly acid than leaves but the range through which change took place was smaller. The alkalinity of the ash was essentially constant save for the observation at noon; it is doubtful that the small increase at that time has significance.

Organic solids and ash were both satisfactorily constant for the four plants as was total nitrogen. The nitrogen content of the stem was appreci-

ably greater than that of the leaf but almost half of it was nitrate nitrogen. Of the balance, about equal parts consisted of protein nitrogen and soluble nitrogen other than nitrate. In this plant, unlike tobacco, nitrate nitrogen is apparently accumulated in the stem; only small amounts were present in the leaf (fig. 2).

Figure 8 shows the behavior of organic acids and carbohydrates. Total organic acids of the larger plants diminished slightly during the day and
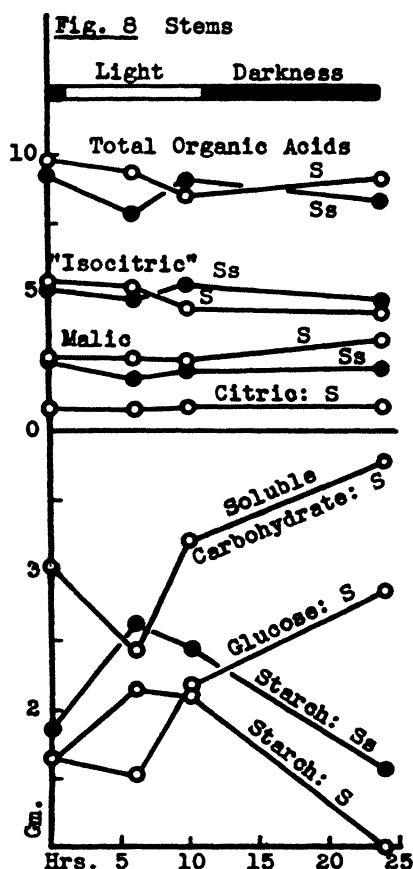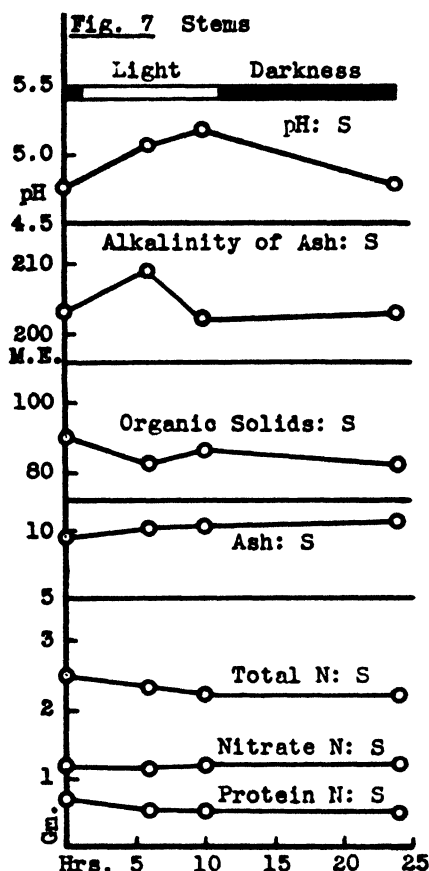
Figs. 7 and 8. Composition of stems of *Bryophyllum calycinum* as influenced by light and darkness over a period of 24 hours. Data expressed in terms of 1 kilogram of fresh weight of tissue. The symbol S refers to stems of large plants; Ss to stems of small plants.

increased to nearly the initial level at night but the relative magnitude of the change was too small to make it certain that this is an example of true diurnal variation. In the smaller plants, there was indeed a drop during the morning, but a high value for "isocitric acid"[4] in the sample collected in the afternoon suggests that increase in this component may have occurred.

[4] Although isocitric acid has recently been identified in the stem tissue of Bryophyllum in this laboratory, the component designated "isocitric acid" in this tissue is determined by subtracting the sum of the malic, citric, and oxalic acids from the total organic acids. Accordingly the presence of still other acids is not excluded. See (9) for a discussion of this point.

The data for malic acid in both large and small plants show no clear evidence of change and it may be concluded that, at least as far as the present experiment is concerned, the stem tissue of Bryophyllum plants does not undergo significant diurnal variation in malic acid. Citric acid also remained constant.

On the other hand, there were variations in the carbohydrates that were obviously significant. Starch increased markedly in the stems of both sets of plants during the day, in fact by 30% or more of the early morning value, and fell well below the initial value during the night. Much of the loss at night can be satisfactorily accounted for by the increase in glucose although the accumulation of starch during the day took place during a period when glucose remained nearly constant, a possible evidence of transport from the leaves. Both sucrose and unfermentable carbohydrate (not plotted) were low initially and changed very little so that the major changes consisted essentially of alterations in the relative concentration of starch and of glucose. There is no indication from these curves of substantial disappearance of carbohydrate components from the stem tissue during the night as was the case in the leaves and, accordingly, the data for the carbohydrates are in essential agreement with the conclusion already drawn; namely, that there is no convincing evidence for the presence of the crassulacean type of metabolism in the stem tissue of the Bryophyllum plant.

There remains to be explained, however, the systematic change in the pH. This followed a pattern entirely consistent with the view that stem tissue shares to a moderate extent in the diurnal change in acidity of the leaf tissue in spite of the fact that the acids in the stem did not change detectably in concentration. However, change in pH can equally well be accounted for in terms of alteration in the acid-base relationships. In view of the failure of the alkalinity of the ash to conform with the change in pH, this alteration could scarcely arise from changes in the relationships with inorganic components. Accordingly, in the absence of evidence, it may be assumed that the change in pH is the result of a temporary increase in organic base during the period of illumination followed by a decrease at night.

Discussion

The present data confirm and in certain details extend previous information on the diurnal behavior of *Bryophyllum calycinum*. As has long been held, variation in acidity can be largely accounted for in terms of progressive alterations in the concentration of malic acid in the leaf tissue, and citric acid shares to a moderate extent in these changes. Isocitric acid, although it is the second most important component of the leaves collected in the early morning and the predominant acid in leaves collected in the afternoon, did not, in the present experiment, undergo parallel systematic changes in concentration; it followed, rather, an irregular course.

There is every reason to assume that soluble carbohydrates are concerned in the reactions that take place, but the observed changes in concen-

tration of these components were minor ones; as has been pointed out by WOLF, it is starch which increases markedly when the acids diminish and which decreases when the acids are resynthesized. Nevertheless, on chemical grounds, it is necessary to assume that the soluble carbohydrates, especially glucose, behave as intermediates in the series of chemical reactions so that the accumulation or diminution of the soluble carbohydrates is a function of the relative velocities of these intermediate reactions. Clear demonstrations only of the accumulation or diminution of the respective endproducts can be obtained.

Present day views on the metabolism of carbohydrates in living tissues, especially in the animal tissues that have been chiefly studied, suggest that carbohydrates are linked with organic acids by means of a series of enzymatic reactions most of which are reversible equilibria. Respiration in pigeon breast muscle as well as in many other tissues is specifically accounted for in terms of the Krebs tricarboxylic acid cycle and the tendency today is to invoke this fundamental scheme for the oxidation of carbohydrates in explaining many other biochemical phenomena. CHIBNALL (4) suggested in 1939, shortly after the scheme was originally proposed, that respiration in plant tissues may well be accounted for along similar lines, and the cycle has also served in speculative attempts to deal with some of the phenomena of amide metabolism in plants (13).

Crassulacean metabolism is clearly a further case upon which light may be shed by the Krebs tricarboxylic acid cycle. Even in the absence of specific information on the enzyme systems present in Bryophyllum leaves, it is obvious that the transformation of starch to organic acids at night involves oxidative reactions and there is a possibility that these reactions undergo at least partial reversal when the leaves are illuminated.

The diurnal variation in the concentration of protein in Bryophyllum leaves and the reciprocal relationship between protein and the concentration of the soluble nitrogen introduces into the general problem a new factor that may possibly be of great significance. Diurnal variation in leaf protein seems rarely to have been studied. Those investigators who have examined the nitrogenous composition of leaves throughout a single day appear to have restricted their analytical examinations for the most part to the total nitrogen. CHIBNALL (3), however, studied the protein content of runner bean leaves collected at nightfall and in the early morning and demonstrated that there was a fall in protein during this period of 1.8% of the evening value, and a parallel fall of 9% in non-protein nitrogen and of 2.5% in total nitrogen. These results were interpreted in terms of translocation of protein digestion products away from the leaf during the night. The change is that to be anticipated in a rapidly growing plant.

The behavior of the protein of Bryophyllum leaves appears to be something entirely different. The data, like those of CHIBNALL, are expressed in terms of concentration in the fresh weight of the tissue but they refer to the whole of the leaflets (i.e., the younger leaves) of a single plant rather

than to the relative composition of opposite members of pairs of leaflets one of which was collected at night, the other in the morning. The phenomenon is a major one; it is not a measure of the small quantity of nitrogen contributed by the leaves in elaborated form to the stem and roots in order to care for their requirements for growth. It concerns changes in the relative concentrations of protein and soluble nitrogen within the leaflets themselves during a period of 24 hours, these changes consisting of transformation into a soluble form (under the conditions of the analysis) of about one-third of the leaf protein *during the day* followed by the reverse transformation during the night. The correlation in periodicity of this phenomenon with the diurnal variation in the organic acids and the starch inevitably raises the question of a possible connection between them. Unfortunately, too little is known about the metabolism of the proteins of plant leaves in general to warrant positive statements. It seems unlikely that diurnal variation of this order of magnitude should have escaped observation hitherto if it is a common phenomenon. That this behavior should occur only in Bryophyllum is equally unlikely; it should certainly be looked for in other crassulacean plants. However, it may be pointed out that the energy requirements of the several over-all reactions are consistent with each other. Protein synthesis takes place during the night when starch decomposition and oxidation of the monosaccharides to organic acids may be supposed to supply the necessary energy; protein decomposition takes place during the period when a supply of energy is available from outside for the synthesis of starch and the transformation of the organic acids presumably in part to carbohydrates. The relationships among the numerous equilibria involved are manifestly extremely complex; that displacements in one direction or the other should occur is not surprising but attempts to assign a reason for such displacements are at present premature.

Summary

The leaves were picked from *Bryophyllum calycinum* plants successively before daybreak, at noon, and at sunset, and again the following morning before daybreak. The large simple basal leaves and the leaflets of the upper compound leaves were kept separate. The stem with attached petioles was likewise harvested. Each sample was dried at 80° C. immediately after collection and determinations were made of organic and inorganic solids, pH, protein, soluble nitrogen, nitrate nitrogen, and of the individual organic acids and carbohydrates. The data were computed in terms of concentration per kilogram of fresh tissue weighed at the time of collection.

The classical phenomena of decrease in organic acids during the day and increase at night were observed and the change was found to arise largely from alterations in the concentration of malic acid although citric acid shared to a moderate extent. Isocitric acid did not change in concentration in a similar regular progressive manner. Only a trace of oxalic acid was present and no significant change was detected.

Starch was synthesized during the day and the quantity that disappeared at night was closely similar to the increase in the quantity of organic acids. Soluble carbohydrates, especially glucose and sedoheptose, varied between moderate limits, the behavior of glucose being consistent with the view that it is an intermediate in the reactions whereby starch was presumably converted to organic acids at night.

The protein of the leaflets underwent an extensive transformation during the day to products that were determined as soluble organic nitrogen; this reaction was reversed during the night.

The behavior of the stem tissue was different from that of the leaves in that only small changes of most of the components occurred. Although there was a moderate progressive change in pH similar to that in the leaves, there was no significant change in malic acid. The concentration of starch decreased substantially at night but there was a simultaneous increase in glucose so that the net change was small. It was concluded that the stem tissue of *Bryophyllum calycinum* does not display the classical phenomena of crassulacean metabolism to a significant extent.

Although specific interpretation of the reactions is impossible in the absence of information on the enzyme systems present in the leaves of this plant, it is probable that the disappearance of starch at night coupled with the increase of malic and, to a lesser degree, of citric acid are the result of oxidation of carbohydrates by mechanisms allied to those of the Krebs tricarboxylic acid cycle. To what extent the synthesis of starch and the disappearance of organic acids during the day may represent a reversal of the reactions that occur at night is not evident from the present data.

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THE PHYSIOLOGICAL ACTION OF 2,4-DICHLOROPHEN-OXYACETIC ACID ON DANDELION, *TARAXACUM OFFICINALE*[1]

LOWELL W. RASMUSSEN

(WITH SIX FIGURES)

The recent introduction of the use of synthetic growth regulating substances as herbicides is giving new stimulus to weed control activities. Because such substances are effective in low concentrations, killing plants through direct effects within the plant system and not seriously damaging the soil, they are especially desirable herbicides. The compound 2,4-dichlorophenoxyacetic acid (2,4-D) is the most widely used growth substance for weed killing purposes at the present time, and it has been shown by several workers (**6, 13, 14, 20**) to have selective action, killing most broad leaf species, while grass species are more or less resistant.

Plants which are susceptible to the action of 2,4-D show various morphological and histological responses (**7, 23, 25**) suggesting that physiological reactions of the cells are affected. Further evidence of effects on the physiological activity of the plant is indicated by the decrease in the dry weight of treated plants (**5, 16, 21**) which suggests the depletion of food reserves and possibly an increase in the respiration rate. A temporary increase followed by a decrease in the sugar content has been noted in annual morning glory [*Ipomea lacumosa* (**16**)] and in bindweed [*Convolvulus arvensis* (**12**)] as a result of 2,4-D applications. A similar response occurred in bean leaves (**17**) treated with alpha-naphthaleneacetic acid. The reserve carbohydrates of plants are rapidly depleted after treatment with 2,4-D (**16, 21, 25**) and the same effect may be caused by applications of other growth substances (**1, 15, 17, 18, 22**). Increased respiration has been shown also to follow applications of 2,4-D (**5, 21**) or indoleacetic acid (**19**), but none of these responses has been satisfactorily established as a direct cause of the death of treated plants.

The common dandelion (*Taraxacum officinale*) is readily killed by 2,4-D, and has been used in this study in an attempt to determine the physiological reactions involved in the herbicidal action of the compound.

General methods

Varying spray treatments with 2,4-D preparations were applied to heavy stands of dandelion plants in bluegrass lawns. One series of tests was run in August and September of 1945 and duplicated in June and July of 1946. Four treatments consisting of control and light (120 ppm.), medium

(480 ppm.) and heavy (1920 ppm.) applications of 2,4-D in Weedone were made in randomized blocks with 4 or 5 replications. The light applications killed 10 to 15% of the dandelion plants; the medium and heavy treatments were about equal with kills of 90 to 100%.

Root samples were taken at the time of treatment and 5, 10, 15, 20, and 25 days afterward. A stratified method of sampling was used in which each plot was divided into four sections, each containing six sampling positions. At each sampling date roots were dug from one randomly designated position in each section within a plot and composited to make up the plot sample of at least 50 grams. Roots that were dead or that showed appreciable decomposition were not used. The samples were protected from drying and then taken to the laboratory, washed, and quickly killed in boiling 80% alcohol.

Two other blocks of plots were used; one in the summer of 1946 for additional studies of the effect of 2,4-D on root respiration, and one in the fall to compare the actions of kerosene and sodium chlorate with 2,4-D. Randomization and sampling procedures were the same as in the main experiments.

Extraction of sugars and non-protein nitrogen in the samples for chemical analyses was completed with 80% alcohol. Methods given by LOOMIS and SHULL (12) were followed in clearing the sugar solutions with neutral lead acetate and estimating sugars by the Munson-Walker-Bertrand method. Sucrose was hydrolyzed with invertase. Dextrin and levulins were extracted with hot water and estimated by the method given by LOOMIS and SHULL (12) with correction for levulose lost in dextrin hydrolysis. Starch was not present, and hemicellulose determinations were not made. Total nitrogen in the alcohol extract, by the reduced iron method, is reported as alcohol-soluble or non-protein nitrogen, and that of the residue as protein nitrogen.

Respiration measurements were made by sealing 50-gm. samples of roots in special mercury-sealed, one-liter flasks developed in this laboratory, and analyzing samples of gas from the flask after 15 to 16 hours. Volumes were corrected to s.t.p. and calculated as milliliters CO_2 produced per kilogram dry root substance per hour.

The effect of 2,4-D on the chemical composition of dandelion roots
METHODS

The changing composition of dandelion roots as influenced by 2,4-D applications at three concentrations was determined over a period of twenty-five days after treatment. None of the roots showed signs of splitting as a result of any increased growth which might have occurred. The root populations of the plots under the medium and heavy treatments were noticeably reduced because many plants were completely killed by the time of the last two samplings. Consequently, the samples on which composition studies were made consisted only of those roots which had resisted the action of the

treatment enough to be still present. When the twenty- and twenty-five-day samples were taken, most of the top growth appeared dead under the medium treatment and only a slight trace of life in the tops was found under the heavy treatment, however, some of these plants which appeared to be dead, even down into the crown, still had roots which appeared normal after the crown was removed.

The results of the two main experiments, run in 1945 and 1946, were very similar; we have, therefore, conserved space by showing data only for the latter season.

RESULTS

REDUCING SUGARS.—Reducing sugars are formed by plants during photosynthesis or by digestion of reserves, and are utilized in respiration or as building materials in the synthesis of protoplasm, cell walls, and storage forms. Any change, therefore, in the quantity of these sugars present as a result of 2,4-D application indicates the possible reactions that are being affected. The reducing sugars present in 100 gm. of fresh dandelion roots from the 1946 tests are shown in figure 1 as percentages of the fresh weight.

The percentage of reducing sugars in the roots before treatment was slightly higher in 1945 than in 1946, and the reductions shown in figure 1 at 20 days started at 10 or 15 days. In both seasons treatments with 2,4-D caused an early rapid increase in reducing sugars followed by a decrease. A large increase in the reducing sugar content of roots suggests an accelerated hydrolysis of the polysaccharide reserves. The later decline might result either from a slowing down of the hydrolytic action or an exhaustion of the reserve carbohydrates.

SUCROSE.—The sucrose content of the 1946 roots and the changes resulting from the treatments are plotted as percentages of the fresh weights in figure 2. The initial sucrose content of the roots was about 1% higher in the 1946 samples taken in early summer than in the late summer samples of 1945. In both tests the 2,4-D applications caused a slight decrease in sucrose while the controls were showing an increase.

POLYSACCHARIDES.—The polysaccharide reserves of dandelion roots are mainly a mixture of dextrins and levulins, as shown by LOOMIS (11) and verified by tests of our material. Dextrins constituted 2 to 3% and levulins 6 to 10% of the fresh weight of the freshly dug, untreated roots; both fractions being somewhat higher in 1946 than in 1945. Dextrin percentages dropped to about half of the control values with medium and heavy 2,4-D treatments. Percentage losses of levulins were comparable, with total losses correspondingly greater. The 1946 data for levulins are plotted in figure 3. The light treatment, which killed only a small percentage of the plants, gave the smallest levulin and dextrin losses, but considerable quantities of polysaccharide reserves were left in the heavier treatments which gave 90 to 100% kills. Disintegration of the dead roots was so rapid that it was not possible to obtain samples at what might be considered the death point.
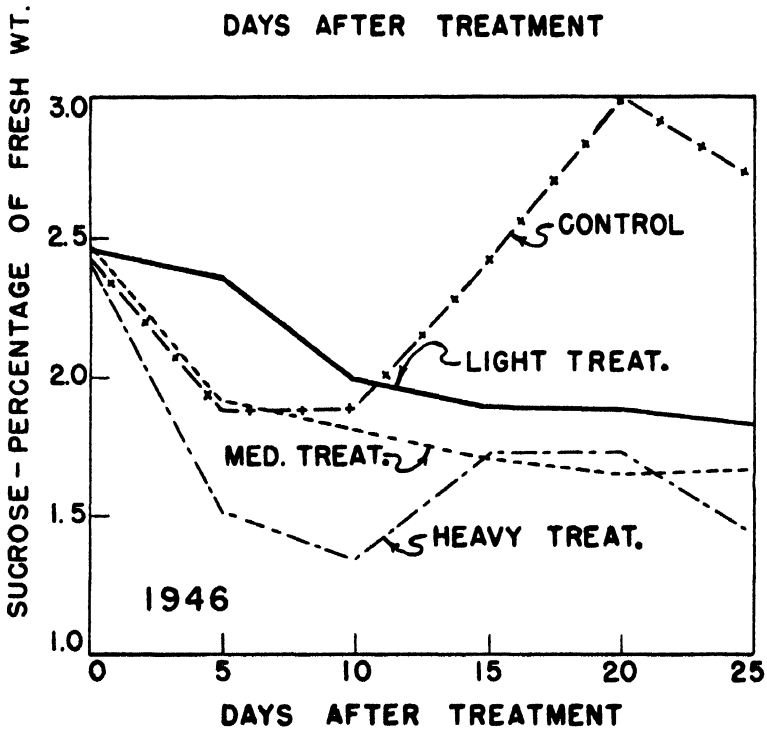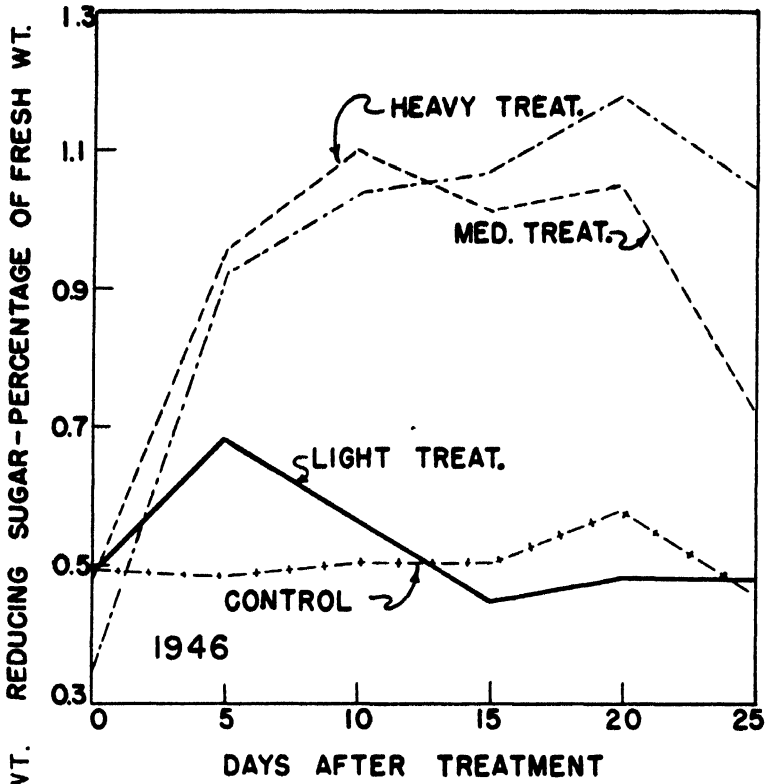
FIG. 1. (Above) Percentages of reducing sugars in control and treated dandelion roots—fresh weight basis.

FIG. 2. (Below) Percentages of sucrose in control and treated dandelion roots—fresh weight basis.

Plants were dying rapidly in 15 days, however, and by 25 days it was diffi-
cult to find enough roots for samples. It seems to be a fair assumption,
therefore, that the roots died before their reserves were exhausted. This
conclusion is supported by the data for total reserves (sucrose, dextrins,
and levulins) shown in figure 4.
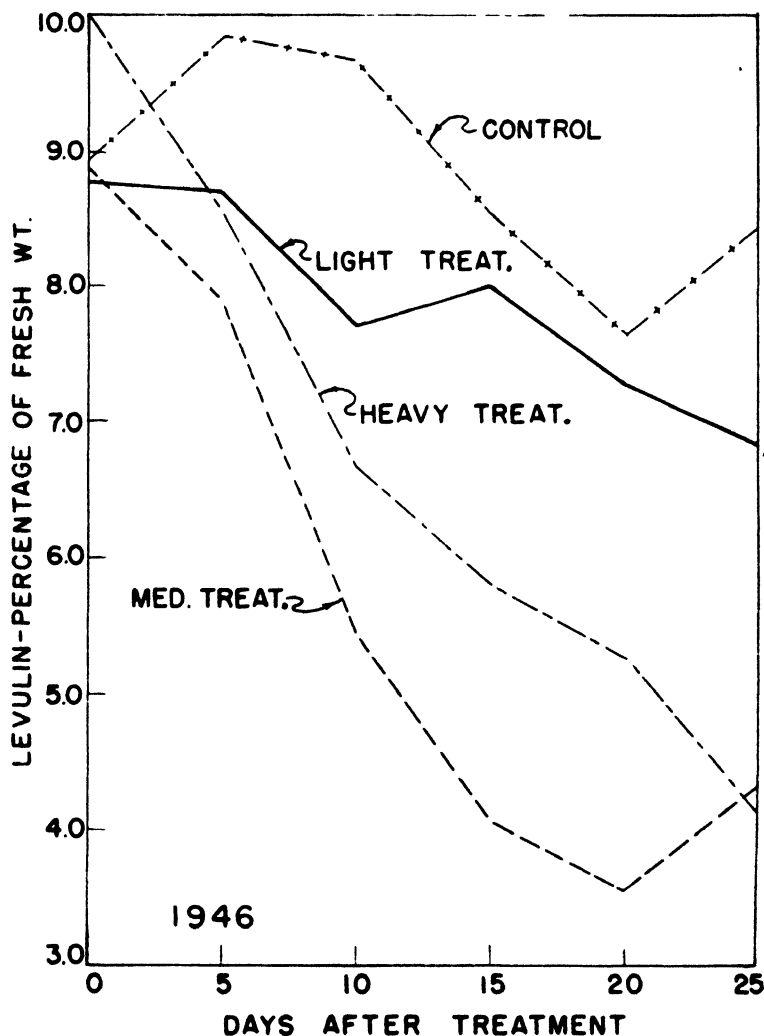
FIG. 3. Percentages of levulin in control and treated dandelion roots—fresh weight
basis.

The decrease in reserve carbohydrates was very much more than enough
to account for the increase in hexose sugars, which suggests that a large
amount of the sugars resulting from the hydrolysis of reserve foods was
either used up in respiration or in the synthesis of non-carbohydrate mate-
rials or both.

NITROGEN ANALYSES.—The nitrogen content of plant parts gives an indi-
cation of the physiological reactions that are taking place. A high soluble
nitrogen content indicates synthesis or hydrolysis and may stimulate new

growth. The protein-nitrogen content indicates whether protoplasm is being synthesized or destroyed.

Nitrogen soluble in 80% alcohol (non-protein N) was less than 0.03%
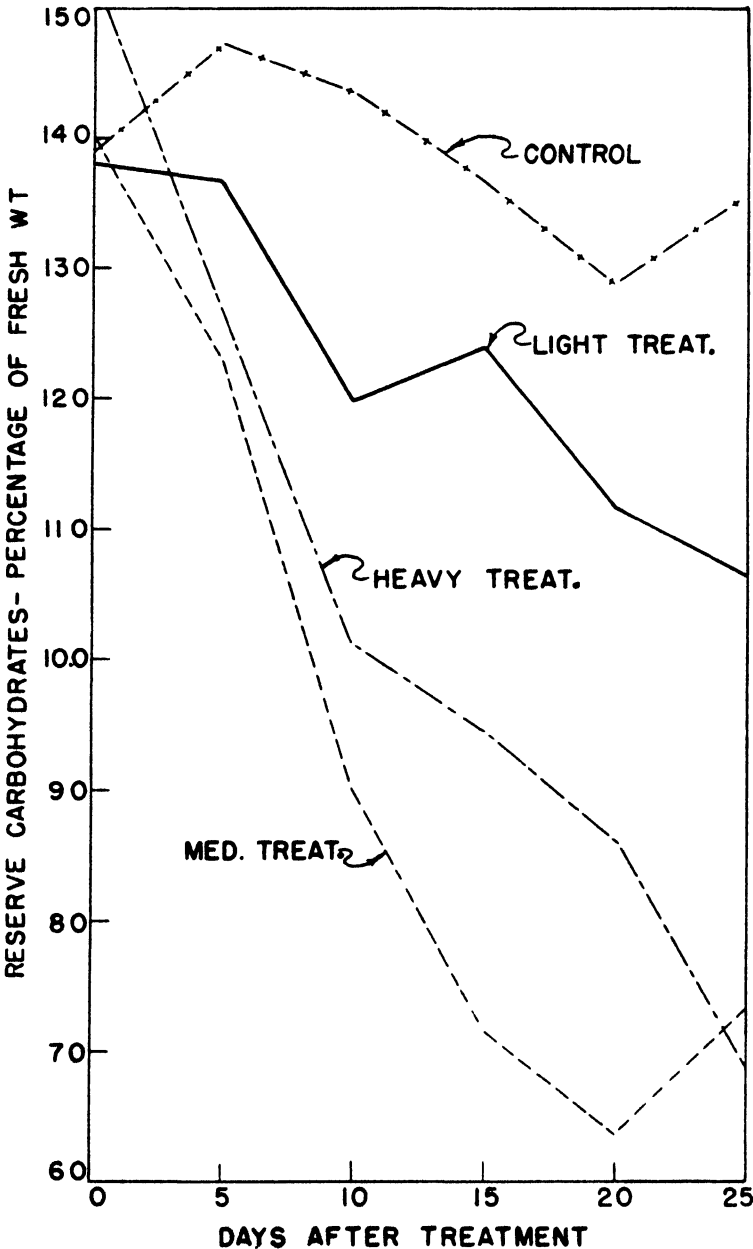
FIG. 4. Percentage reserve carbohydrates in control and treated dandelion roots—fresh weight basis.

in June of 1946 and up as high as 0.08% in August of 1945, with an upward trend in the controls of both series. The effect of 2,4-D treatments (fig. 5) was to raise these percentages by 2 to 5 times, with the heavy treatment tending to show the least effect and the light treatment the most. If soluble

nitrogen is considered to be stimulating for cell division (10), these results suggest that the lighter treatments should have been more stimulating, while the heavier were showing direct toxicity.

The protein nitrogen also showed a tendency to increase during the period of each test, although the light and heavy treaments of 2,4-D did not cause much deviation from the controls. The medium treatment caused the greatest change, showing an accelerated increase which reached a maximum
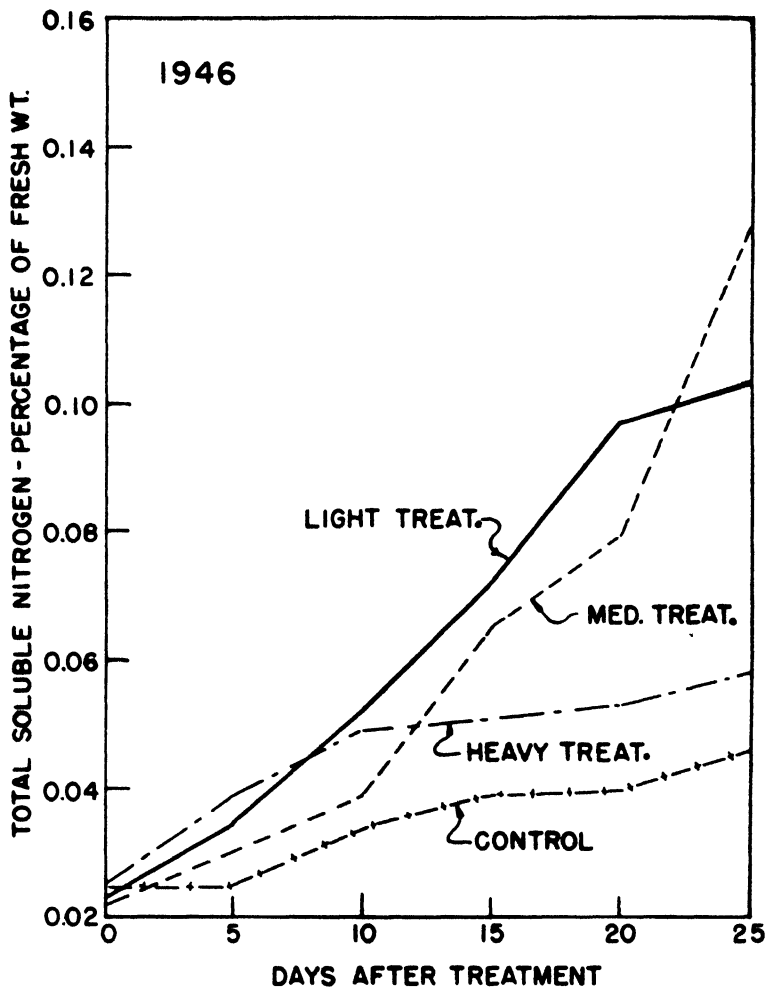
FIG. 5. Percentage total soluble nitrogen in control and treated dandelion roots—fresh weight basis.

fifteen days after treatment. The increase in both protein nitrogen and soluble nitrogen suggests an increased rate of absorption of nitrogen compounds from the soil and the synthesis of amino acids and proteins. It is possible that the increased protein nitrogen was merely an indication of the depletion of the carbohydrate reserves, but the expression of the results on a fresh weight basis would reduce this error, and the small increases in the heavy treatment, which gave the largest carbohydrate losses, suggest that it was not a factor.

The effect of 2,4-D on the respiration of dandelion roots

The rate of respiration in plant tissue is a measure of reaction intensity, and it is affected by both internal and external factors. The application of toxic substances to plants often stimulates an increase, usually followed by a decrease, in respiration while the normal response to a small quantity of growth substance is an increase in respiration which continues at a high rate as long as the reactions are possible (**4, 24**).

The rate of respiration of root samples from the 1946 test, on which chemical composition studies were also made, are shown in figure 6. The treatment with 2,4-D caused an early, rapid increase, with the medium treat-
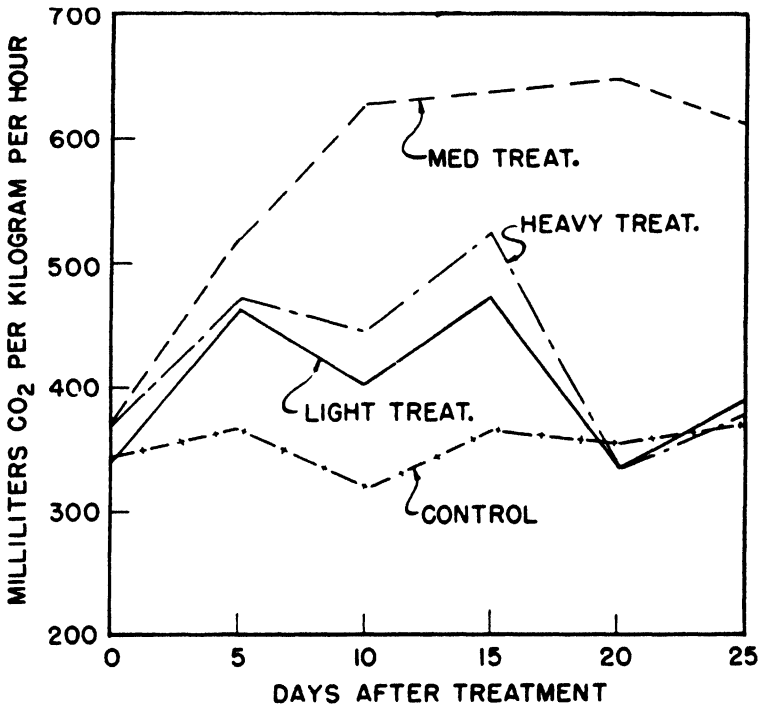
FIG. 6. Rates of respiration in dandelion roots.

ment producing the greatest response, which continued throughout the period of the test. The percentage increases for the plants receiving the medium treatment were 92, 75, 83, and 66 at 10, 15, 20, and 25 days, respectively, after treatment. This increased respiration rate with the medium treatment was concurrent with the increased reducing sugar content (fig. 1), and suggests the possibility of the high sugar content causing the accelerated rate of respiration. The heavy treatment of 2,4-D, however, indicates that the effects on sugars and respiration are two separate processes, as this treatment increased the sugar content considerably throughout the test periods, but increased respiration only slightly for the first 15 days, after which it decreased, even though the sugar content was high.

The results between 5 and 15 days would fit well an hypothesis of a toxic action of 2,4-D, with an increasing stimulation of respiration from light to

medium treatments and a drop with the excess, heavy treatment. The 20-day lows for light and heavy applications would then represent, respectively, the wearing off of the light effect and progressive destruction of protoplasm and enzymes with the heavy treatment. If this hypothesis is accepted it should be noted that the stimulating effect of the medium treatment on respiration continued until near the death of the roots. A second series of experiments with the medium treatment only showed this same effect; respiration was doubled within 10 days after treatment and remained high until the death of the plants. If these reactions are indicators of direct protoplasmic toxicity, as we believe they are, they indicate an action on some part of the cell not directly concerned with respiration.

The comparative action of 2,4-D and other herbicides
METHODS

An experiment was run to compare the effects of 2,4-D, 960 ppm., with kerosene, 200 gal. an acre and sodium chlorate, 475 pounds an acre, when applied in the fall of the year. Kerosene was used because it has been shown by LOOMIS (11) to have selective herbicidal action in killing dandelions in lawns. Sodium chlorate is a commonly used herbicide which has been shown by BAKKE, GAESSLER, and LOOMIS (2) to cause a depletion of the food reserves in bindweed.

The chemical determinations reported for the fall experiments were made on samples taken 4 and 14 days after application of the treatments. The data for the two determinations are shown in table I. The data of the 14-day analyses were analyzed statistically, and the mean significant difference was calculated for comparing the values for the 2,4-D treatment with the others.

TABLE I

CARBOHYDRATE ANALYSES OF DANDELION ROOTS TAKEN 4 AND 14 DAYS AFTER TREATMENT. MEANS OF THREE REPLICATES EXPRESSED AS MILLIGRAMS PER 100 GRAMS OF FRESH ROOTS

TREATMENT	REDUCING SUGAR		SUCROSE		DEXTRIN		LEVULIN	
	4 DAYS	14 DAYS*	4 DAYS	14 DAYS†	4 DAYS	14 DAYS‡	4 DAYS	14 DAYS§
Control	302	140	2733	2688	2060	2598	6833	7916
Kerosene	970	1467	1743	2432	1888	1305	6432	3676
Sodium chlorate	152	457	1382	1767	2225	1912	7991	5834
2,4-D	323	410	1621	1662	1768	1270	5885	3564

* Significant mean difference from 2,4-D 410 ± 286.
† Significant mean difference from 2,4-D 1662 ± 532.
‡ Significant mean difference from 2,4-D 1270 ± 571.
§ Significant mean difference from 2,4-D 3564 ± 1759.

RESULTS

CARBOHYDRATES.—Kerosene caused an increase in reducing sugar to a percentage significantly higher than that for 2,4-D. The 2,4-D treatment in

this fall test did not cause a significant increase in reducing sugar over the untreated controls, differing in this respect from the large increases caused in the summer tests.

At 4 days after treatment the sucrose content was significantly lower in the roots from treated plots compared with control, but there was no difference among the three herbicides. At 14 days, however, 2,4-D and sodium chlorate-treated roots were still lower in sucrose than the control, but the sucrose had increased under the kerosene treatment to a level significantly higher than the other two herbicides, and equal to the control (table I).

Four days after treatment there were no significant effects on the dextrin content of the roots. At 14 days the dextrin content in the roots from plots treated with the herbicides was significantly lower than in the controls. The dextrin content of the 2,4-D-treated roots was lower than the sodium chlorate-treated roots; kerosene and 2,4-D caused comparable changes in the dextrin content.

TABLE II

NITROGEN ANALYSES OF DANDELION ROOTS TAKEN 4 AND 14 DAYS AFTER TREATMENT. DATA ARE MILLIGRAMS PER 100 GRAMS OF FRESH ROOTS

TREATMENT	SOLUBLE NITROGEN		PROTEIN NITROGEN	
	4 DAYS	14 DAYS*	4 DAYS	14 DAYS†
Control	171	207	171	173
Kerosene	143	189	196	222
Sodium chlorate	216	197	193	200
2,4-D	134	100	184	198

* Significant mean difference from 2,4-D 100 ± 68.
† Significant mean difference from 2,4-D 198 ± 11.

There were no significant differences in the levulin content four days after treatment although the 2,4-D treatment showed a lower quantity than the other treatments. The levulin content decreased for each of the herbicidal treatments to a level significantly below the control by the fourteenth day. There was no difference in the effect of 2,4-D and kerosene on the levulin content, but the sodium chlorate treatment was significantly higher.

NITROGEN.—The total soluble nitrogen content of the roots and the effect of the treatments on this component are shown in table II. The differences four days after treatment were not significant, as indicated by an analysis of variance. However, by 14 days after treatment the quantity of soluble nitrogen in the roots from plots treated with 2,4-D had decreased to a level significantly below all of the other treatments. This response is different from the effect produced by the lighter applications in the summer tests in which the soluble nitrogen increased in the roots.

The herbicidal treatments caused an increase in the protein nitrogen of the roots, as shown also in table II. The differences, however, were small and were not significant until 14 days after treatment. The 2,4-D and

sodium chlorate gave the same response, while kerosene caused an increase significantly higher than 2,4-D.

RESPIRATION RATES.—When root samples were taken for chemical analysis, enough extra roots were dug to make respiration rate measurements on each treatment with two replicates. The mean rates of respiration and the respiratory quotient for each treatment are given in table III. Both 2,4-D and kerosene caused large increases in the rate of respiration, but the type of materials being utilized or some steps in the process were evidently different, as indicated by the higher respiratory quotient for the kerosene treatment.

In this test the 2,4-D treatment caused a large increase in respiration, but did not cause an increase in reducing sugars (table I), which indicates again that the respiration rate of the 2,4-D treated roots was not correlated with sugar content.

TABLE III

RESPIRATION RATES EXPRESSED IN MILLILITERS CO_2 PER KILOGRAM OF DRY ROOT MATERIAL PER HOUR, AND THE RESPIRATORY QUOTIENTS OF DANDELION ROOTS—1946

TREATMENT	DAYS AFTER TREATMENT		RESPIRATORY QUOTIENT
	4	14	
Control	405	408	0.94
Kerosene	934	869	1.24
Sodium chlorate	462	645	0.96
2,4-D	682	1059	0.97

Discussion

The reducing sugar content of the roots from treated plants was increased but the sucrose content was decreased more than enough to account for the rise in reducing sugar, the net effect being a decrease in total sugars. The reserve polysaccharides, dextrin and levulin, decreased rapidly after the 2,4-D treatment. These changes in the carbohydrates of the roots are in agreement with those reported by SMITH, HAMNER, and CARLSON (21) for bindweed roots, and MITCHELL and BROWN (16) for annual morning glory. Other growth substances produce similar effects according to several investigators (1, 15, 17, 18, 22). The increase in reducing sugars is probably the result of an accelerated hydrolysis of reserve carbohydrates. Depletion of the total carbohydrate reserves was marked, but it is doubtful that the plants were killed by starvation, since the greatest decreases amounted to only 68% of the total in 1945 and 49% in 1946. The carbohydrate losses caused by the three concentrations of 2,4-D after 25 days are summarized in table IV. The milligrams of total carbohydrates lost as a result of each treatment concentration were about the same each year even though the percentage decrease was greatest in 1945. It is interesting to note that increasing the concentration of 2,4-D four times (120 to 480 ppm.) caused a twofold decrease in carbohydrates, while a further concentration increase of four times

(480 to 1920 ppm.) caused only a slight additional decrease, showing that the lower concentrations of 2,4-D gave the greatest physiological response in relation to the quantity applied.

The decrease in carbohydrate content of the roots is reflected in a decrease in the total dry weight of the roots, and a comparison of these decreases gives an indication of the reactions involved. A decrease in dry weight equal to the decrease in carbohydrates suggests utilization of the carbohydrates in respiration, while a decrease in dry weight less than the decrease in carbohydrates would indicate some synthesis of non-carbohydrate materials. We found in our experiments that the decrease in dry weight was practically equivalent to the loss in carbohydrates, indicating that very little if any carbohydrate was converted into non-carbohydrate

TABLE IV

DECREASE OF RESERVE CARBOHYDRATES CAUSED BY 2,4-D, 25 DAYS AFTER TREATMENT

TREAT-MENT— 2,4-D	SUCROSE		DEXTRIN		LEVULIN		TOTAL	
1945								
	mg.	%	mg.	%	mg.	%	mg.	%
Light	983	36	310	19	1586	29	2880	29
Medium	1446	54	691	42	3314	61	5452	56
Heavy	1857	69	879	53	3942	73	6679	68
1946								
Light	879	32	430	18	1613	19	2921	22
Medium	1062	39	1082	45	4098	49	6241	46
Heavy	1284	47	1063	44	4331	51	6677	49

material. Consequently little or no growth or differentiation should be shown, and in confirmation there were no indications of growth in the dandelion roots at any time during the tests. In this respect the response of dandelion roots is different from that of bindweed and sow thistle roots reported by TUKEY et al. (25) who observed large increases in root and rhizome diameters accompanied by splitting. They also found that starch disappearance was correlated with new cell growth. It is probable that detailed histological studies of the dandelion root would reveal at least some growth or differentiation, since such a response in other plants has been shown by several investigators (3, 8, 9, 23). Apparently, however, the growth responses are small in the dandelion roots.

The 2,4-D applications approximately doubled the respiration rates of the dandelion roots. The heavy application, which was the most rapidly toxic to the plants, caused only a temporary increase in respiration which

dropped back to normal or below after 15 days. The medium treatment, which was sufficiently toxic to kill most of the dandelions, caused a large increase in respiration which remained high throughout the test, showing only a slight drop after 20 days.

Since the decrease in carbohydrates was apparently all due to oxidation, the question was raised as to whether the increased rate of respiration was sufficient to account for the carbohydrate loss. To accurately determine the balance between the rate of respiration and carbohydrate loss would require information on the rate of photosynthesis, and such data were not obtained. The data we have, however, give some indication of the balance. The mean daily loss of carbohydrates was determined and the rate of respiration necessary to account for the loss was calculated. It was estimated that if the light and medium treatments caused a decrease in photosynthesis of one third, then the observed rate of respiration would account for the loss of carbohydrate. The heavy application would have had to practically stop photosynthesis in order for the observed rate of respiration to account for the carbohydrate loss. Since the leaves were quickly killed by this spray this assumption is not unreasonable.

A study of the effect of 2,4-D, kerosene, and sodium chlorate on the composition and respiration rate of dandelion roots showed that 2,4-D and sodium chlorate had the same effect on reducing sugars, while kerosene caused a much greater increase in reducing sugars and only a slight decrease in sucrose. Kerosene and 2,4-D caused similar decreases in dextrin and levulin, while sodium chlorate was less effective. A check on May 20, 1947 of the plots of the 1946 fall test showed nearly a complete eradication of the dandelions in the 2,4-D plots, and practically equal kills with kerosene, while the sodium chlorate treatment was the least effective. The grass was nearly all killed by the chlorate, slightly injured by the kerosene, but not visibly injured with 2,4-D.

The striking effects of the differential herbicides, 2,4-D and kerosene, were increased digestion and respiration of reserve carbohydrates. The plants did not, as early popular accounts have suggested, "grow themselves to death"; neither did they starve to death, and the respiration should in our opinion be classed as a symptom of specific toxic effects rather than the direct cause of injury. The resistance of the grasses and of some other plants to these differential sprays suggests that the toxicity of these substances may be directed toward certain cytoplasmic compounds, probably proteins, which vary between families and to a lesser extent between genera or species.

Summary

1. The reducing sugar content of dandelion roots increased rapidly following applications of 2,4-dichlorophenoxyacetic acid, but later decreased towards the level of the control. The sucrose of the roots declined slowly after treatment, and the dextrin and levulin contents decreased rapidly.

The decrease of carbohydrate reserves was considerably more than enough to account for the increase in reducing sugar. It was found that the loss could be roughly accounted for by the increased rate of respiration, indicating little if any utilization of sugars for growth response.

2. Respiration rates increased with the 2,4-D; the low concentration giving only a temporary stimulus with a return to normal, possibly because of the small quantity of 2,4-D, while the medium concentration gave the greatest increase which remained high as long as the roots were alive. The highest concentration caused a temporary increase followed by a decrease.

3. A comparative test of 2,4-D with kerosene and sodium chlorate showed that 2,4-D and kerosene were equally effective in decreasing the carbohydrate reserves and in increasing the rate of respiration, of dandelion roots, while sodium chlorate was less effective. Kerosene and 2,4-D were approximately equal in eradicating the dandelions with little or no effect on the grass, while sodium chlorate gave slightly less kill of dandelions and killed nearly all of the grass.

4. From the results of the studies it appears that the action on dandelion of 2,4-D in herbicidal concentrations is principally the destruction of carbohydrate reserves, with most of the loss being accounted for by increased respiration. It seems improbable that these effects alone can account for the lethal action of 2,4-D and we have postulated that they are symptoms of direct, specific, protoplasmic toxicity rather than causes of injury.

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GROWTH OF THE DANDELION SCAPE[1]

MARIAN DELLERS CHAO

(WITH SIX FIGURES)

The growth of the flowering stalk of dandelion, *Taraxacum officinale,* is of considerable interest because of the presence of two distinct periods of rapid growth. The last of these precedes and accompanies the maturation of the fruit, and represents a resumption of rapid growth in what would normally be considered a mature organ.

MIYAKE (**8, 9**) showed that growth of the dandelion scape may be divided into three phases: the first a fairly rapid, uniform growth, the second a phase of slow growth; and the third, a rapid growth in the upper portions of the organ. SCHMALFUSZ (**10**) showed that mitotic figures were present in elongating scapes up to the stage of shedding ripe fruits and were especially frequent preceding the periods of greatest scape elongation.

As a part of a general study of the mechanism of cell elongation the experiments of MIYAKE and SCHMALFUSZ have been repeated and extended. The data presented were obtained from extensive studies of the regions and periods of cell division and elongation in the dandelion scape, together with studies of cell volume, green and dry weight, and nitrogen content on a per cell basis.

Cell division and enlargement

GROWTH OF SCAPE

A uniform plot of uncut sod with a large proportion of dandelions was used for these studies. The dandelion scapes grew irregularly over a period of 32 days. In the first phase, which occupied about nine days in the material tested and lasted from the first appearance of the scape until the blossom opened, the scapes grew rapidly and uniformly throughout their length. The second phase, a period of slow scape growth, began just before the blossom closed and extended over a period of about twelve days, corresponding to the time of embryo development. The final phase covered an eleven-day period beginning sometime before the opening of the seed head and continuing for several days after all the seeds were dispersed. During this time the scapes again grew vigorously.

The growth curve for the whole scape, based on the average of values for forty typical scapes, is shown (fig. 1a). Whereas the usual growth curve of an organ is S-shaped, this curve has two S-shaped regions with a flat region of slow growth interposed. For comparison the growth curves of the

[1] A portion of a thesis presented to the Graduate Faculty of the Iowa State College in partial fulfillment of the requirements for the degree Doctor of Philosophy. A complete copy of the thesis is on file at the College Library.
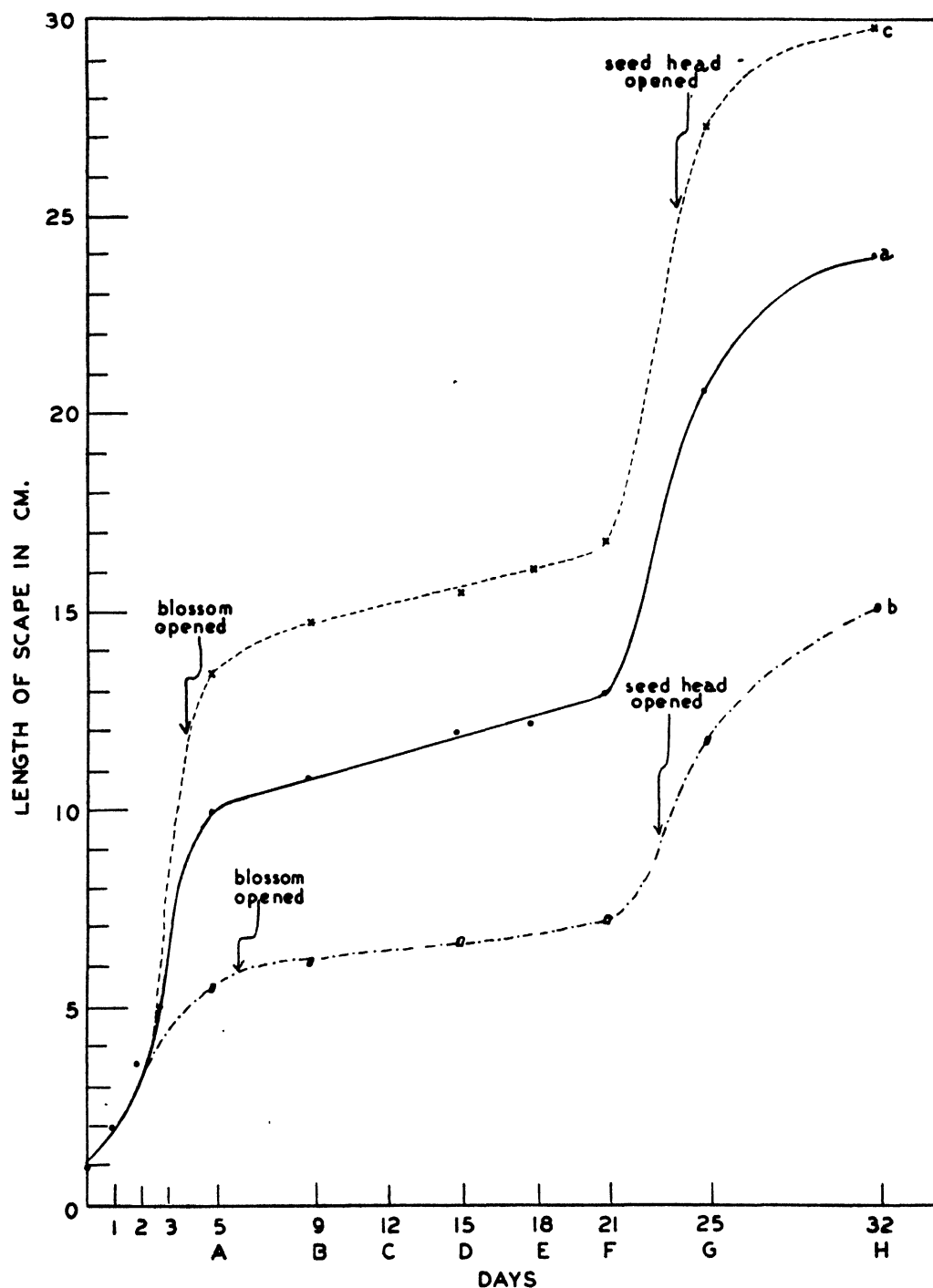
FIG. 1. Growth of the dandelion scape. a. Average scape; b. short scape; c. tall scape.

single scape attaining the smallest final length (fig. 1b) and the single scape attaining the greatest final length (fig. 1c) are also shown. The shape of these three curves is similar and the three phases can be distinguished in each.

CELL GROWTH AND SECTION GROWTH

Bud-scapes averaging 7.26 cm. in length were marked with India ink at 1-cm. intervals for the main growth experiment. Eight times during the period of development the lengths of the sections between markings were recorded. The average cell length in stripped epidermal sections from parallel material, mounted in water, was measured with an eyepiece microm- eter at the same times. Cell length at each stage was determined for sec- tions of the scape immediately under the floral parts (top), center-scape sections (center), and sections at the base of the scape (base). For each section at a given stage, twenty epidermal cell lengths were measured from each of four scapes and the average of these eighty cells used for estimations of changes in cell size and number. In parallel experiments bud-scapes as small as 1 cm. in length were marked with India ink to observe zonal growth, and epidermal sections were measured for cell length.

Data from these experiments are summarized (table I, fig. 2). In the earliest stages, before the bud-scapes were 5 cm. long, cell division was rapid in the upper portions of the scape as shown by the small or negative increase in cell lengths and the doubling in length of the sections. Basal sections showed less cell division; however, the small increase in cell length relative to section length at the 5-cm. stage is interpreted as cell division. At stage "A," blossom open, all three zones grew rapidly and primarily by cell elongation. After.the blossom head closed, the scape grew very slowly. The elongated cells in the sections at the top of the scape underwent an average of one division each, as the average cell length decreased by about half and the scape as a whole showed little elongation. In the middle of the scape cell divisions also occurred, but probably only about half of the cells (the longer ones) divided, and no more than one division occurred in any one cell. At the base of the scape there was even less activity, and prob- ably only scattered cell divisions occurred in the longer cells, as the average epidermal cell length decreased only slowly and to a small extent.

During the last rapid growth of the scape, cell elongation again kept pace with scape elongation, indicating that few or no cell divisions occurred at this time. The most rapid growth took place at the upper end of the scape. The center of the scape showed less growth, and the base showed only a small amount of elongation.

Camera lucida drawings of epidermal cells from the center of typical dandelion scapes at the various stages, show the sequence of events in the cells during scape growth (fig. 3). The small cells seen in the bud-scape (fig. 3A, 3B) elongated until they attained a great length at the open blos- som stage (fig. 3C). After the blossom closed, these extremely long cells began to divide (fig. 3D) and the new cell walls (shown as stippled) were easily distinguishable from the older walls, being less distinct in appearance. Before the seed head opened none of the very long cells was visible, cell divisions having produced two shorter cells for each long one (fig. 3E). As.
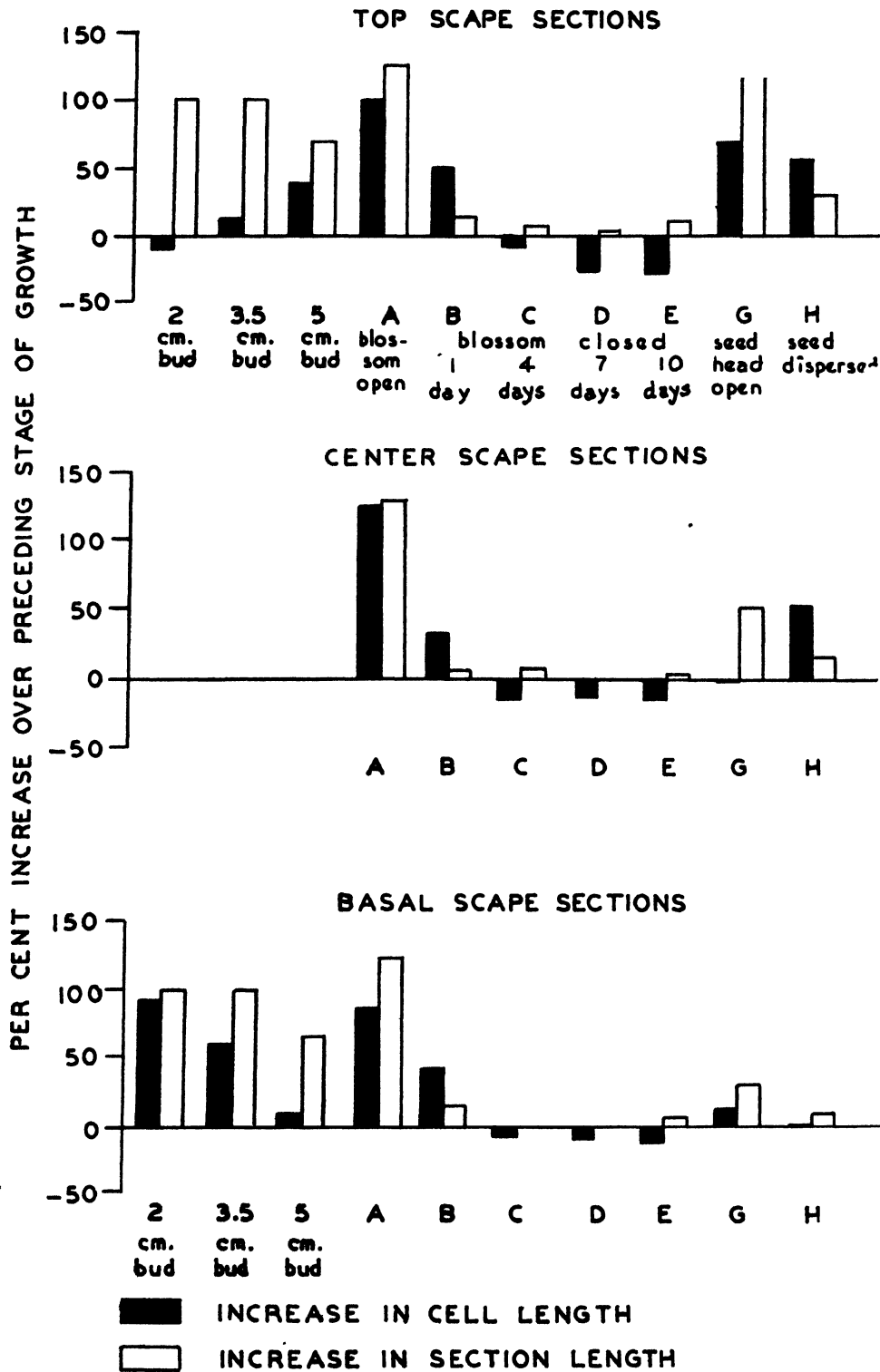
FIG. 2. Elongation of epidermal cells and of marked sections of dandelion scape.

TABLE I

TOTAL ELONGATION AND CELL ELONGATION IN THE DANDELION SCAPE

Growth Stage	Top				Center				Base			
	Section		Cell		Section		Cell		Section		Cell	
	Length (cm.)	Increase* (%)	Length (μ)	Increase* (%)	Length (cm.)	Increase* (%)	Length (μ)	Increase* (%)	Length (cm.)	Increase* (%)	Length (μ)	Increase* (%)
Buds:												
1 cm.	0.1	....	15.7		0.1	....		....	0.1	....	15.7	....
2 cm.	0.2	100	14.0	-11	0.2	100		....	0.2	100	30.1	92
3.5 cm.	0.4	100	16.7	19	0.4	100		....	0.4	100	46.8	55
5 cm.	0.6	50	23.4	40	0.6	50	53.5		0.6	50	50.1	7
Blossom:												
A, just open	1.4	133	46.8	100	1.4	133	123.6	131	1.4	133	90.2	80
B, just closed	1.65	18	70.2	50	1.5	7	163.7	32	1.65	18	130.3	44
C, closed 4 days	1.8	9	66.8	-5	1.6	7	143.7	-12	1.65	0	123.6	-5
D, closed 7 days	1.9	6	50.1	-25	1.6	0	123.6	-14	1.65	0	116.9	-5
E, closed 10 days	2.2	16	36.7	-27	1.65	3	106.9	-14	1.75	6	103.6	-11
F, closed 13 days		....				....	100.2	....		....	110.2	....
Seed:												
G, head open	4.9	123	60.1	64	2.45	48	105.2	-2	2.25	29	116.9	13
H, dispersed	6.3	29	93.2	55	2.9	18	159.7	52	2.4	7	117.3	0

* Increase over previous stage.

the seed head opened (fig. 3F) the cells were again elongating, although they never attained the length they had during the period of blossoming.
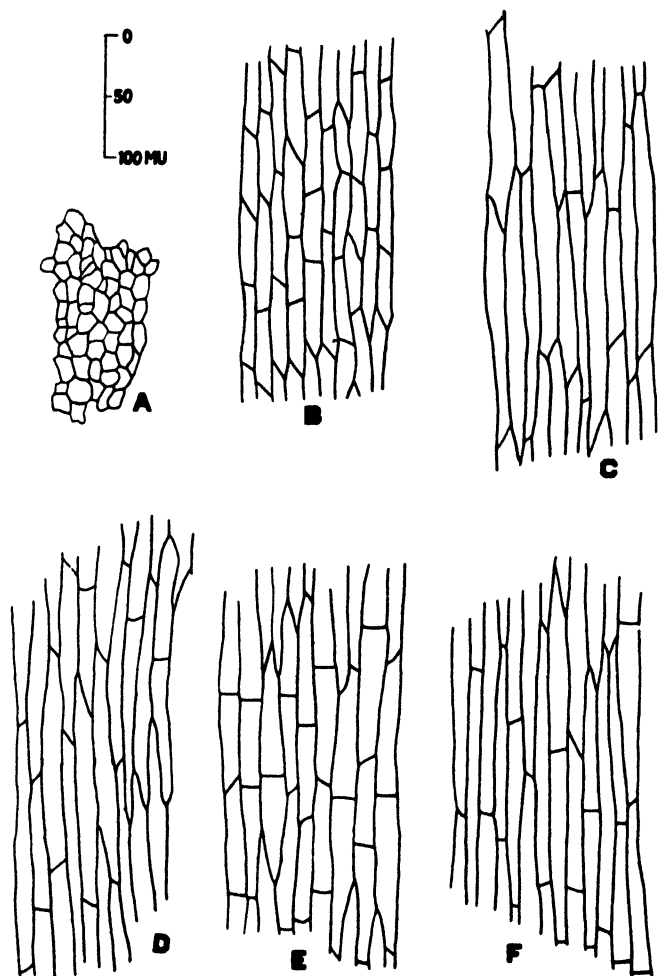
FIG. 3. Epidermal cells from the center of the dandelion scape during growth. Figures were drawn from living material with the aid of the camera lucida. a. Epidermal cells of the 2-cm. bud-scape; b. epidermal cells of the 5-cm. bud scape; c. epidermal cells of the blossom-scape; d. epidermal cells of the scape several days after blossoming; e. epidermal cells of the scape several days before opening of the seed head; f. epidermal cells of the scape of the open seed head.

CELL ELONGATION IN EXCISED SECTIONS

Sections of scape 1 cm. in length grew very satisfactorily in indoleacetic acid–sucrose solutions in Petri dishes, elongating as much as two or three times in a period of several days under favorable conditions. It was unnecessary to use any sterile precautions, since changing the solutions once a day prevented excessive bacterial contamination for as long as four days. A 1% concentration of sucrose was found satisfactory, higher concentrations shortening the number of days during which it was possible to grow unsterile sections of scape without tissue deterioration. Indoleacetic acid gave

best results at 1 p.p.m. to 5 p.p.m. The addition of sources of nitrogen and phosphorus increased contamination and did not accelerate growth of scape material.

Top sections of bud-scapes showed the greatest increase in length in solution, blossom-scapes elongating moderately but not to the same extent, and the older scapes showing even less growth. The relatively great elongation of the bud-scapes was to be expected, since the cells of such scapes were small, and rapid growth of the intact organ normally occurs at this time. The elongation rate of sections of bud-scape in sucrose-auxin solution was comparable to that which occurred on the plant. Elongation of top sections of the scape of the closed flower, "F," did not approach that attained in the plant, sections elongating only 50% under the most favorable conditions of temperature and indoleacetic acid concentration.

TABLE II

ELONGATION OF SECTIONS AND OF EPIDERMAL CELLS OF THE DANDELION BUD-SCAPE DURING 24 HOURS IN A 1% SUCROSE SOLUTION CONTAINING 5 MG. INDOLEACETIC ACID PER LITER. (AVERAGE OF 10 SECTIONS OR OF 80 CELLS)

TEMPERATURE	FINAL LENGTH* OF SECTIONS	FINAL LENGTH† OF EPIDERMAL CELLS	INCREASE IN LENGTH OF EPIDERMAL CELLS
°C.	cm.	μ	%
0.5	1.10	34.7	37.7
5.0	1.13	33.4	32.5
10.0	1.27	28.7	13.9
14.0	1.50	41.7	65.5
20.5	1.60	42.8	69.8
23.0	1.53	45.7	81.3
27.0	1.87	48.5	92.5
31.0	1.60	50.1	98.8
35.0	1.53	34.7	37.7

* Initial length of sections, 1 cm.
† Initial length of epidermal cells, 25.2 cm.

The elongation of sections and epidermal cells of the dandelion bud-scape during 24 hours in solution at various temperatures is shown in table II. These results are typical of three similar experiments. Although cell division occurred in scapes which remained in solution several days, no evidence of cell division was obtained in the 24-hour period, cell elongation being of the same magnitude as section elongation within the limits of variability. Rise of temperature markedly increased the rate of cell elongation. As increase of temperature does not accelerate physical processes to any marked extent, the large increase of cell elongation rates with rising temperatures indicates that chemical reactions are involved.

Nitrogen and dry weight changes

Nitrogen determinations were made by the semi-micro Kjeldahl method, using essentially the procedure proposed by MA and ZUAZAGA (7). Samples for fresh weight, dry weight, and nitrogen determinations were collected on

the same days that cell lengths were studied, and comparable scape material was used. A comparison of total nitrogen with protein and non-protein nitrogen was made on sections from control parts of scapes at all critical stages, using the method outlined by BLANK and FREY-WYSSLING (3) for separation of protein and non-protein nitrogen.
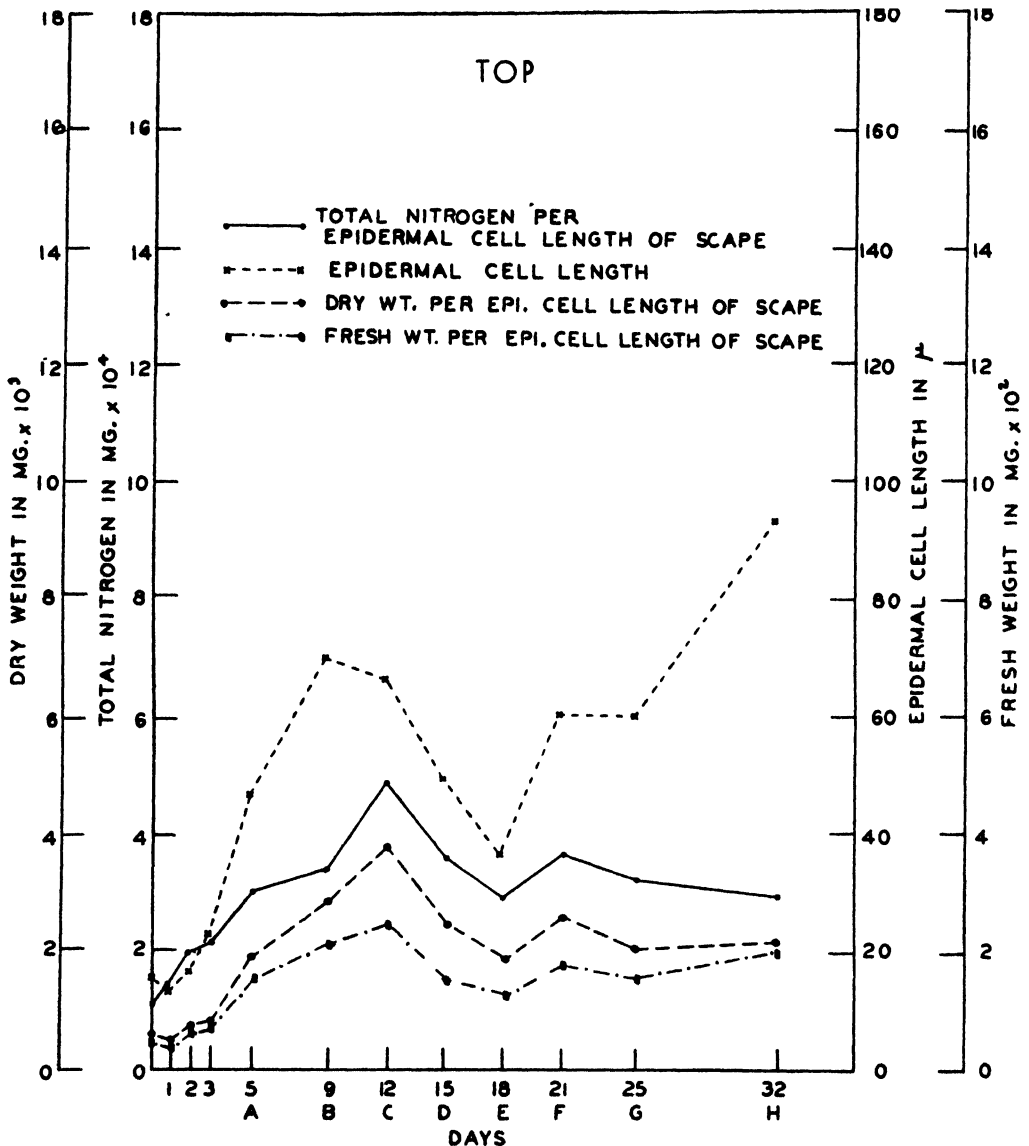
FIG. 4. Total nitrogen, fresh weight, and dry weight, in relation to epidermal cell length at the top of the growing dandelion scape.

The total nitrogen, green weight, and dry weight, changes of three representative regions of the dandelion scape during growth are plotted (figs. 4, 5, 6). In all parts of the scape, total nitrogen in percentage of dry matter was highest in the bud stage, indicating that carbohydrate accumulation was subsequently more rapid than nitrogen accumulation. The amount of pro-

tein nitrogen in the bud-scapes was about 2.5 times as great as the amount of non-protein nitrogen. In the bud the floral parts were developing and the egg cells and pollen were being produced. Nitrogen was undoubtedly moving through the scape into the bud, but nitrogen per epidermal scape cell increased continuously and progressively from the time the scape was
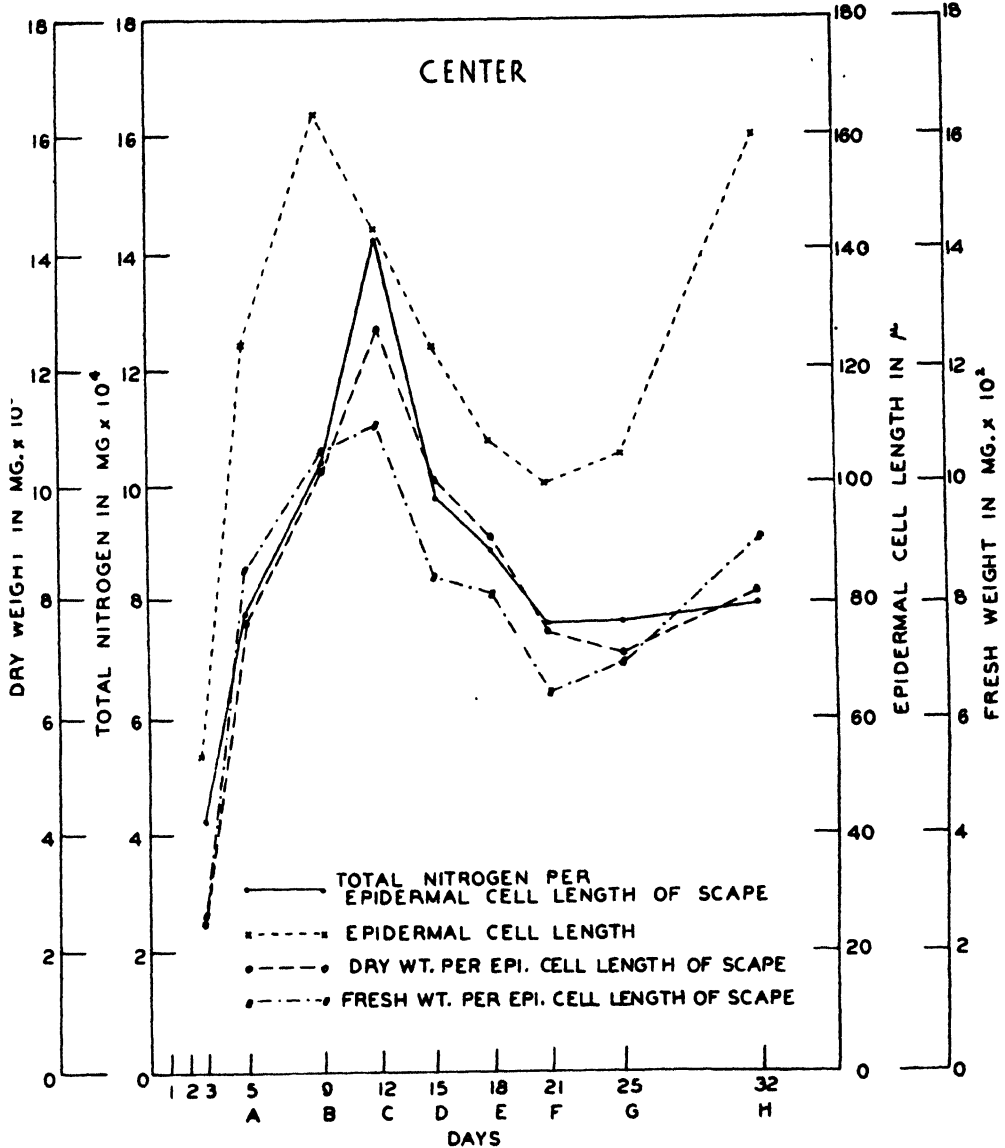
FIG. 5. Total nitrogen, fresh weight, and dry weight, in relation to epidermal cell length at the center of the growing dandelion scape.

first evident until the period of blossoming was well past. The increase of nitrogen per cell was comparable in extent to the rapid increase of epidermal cell length which occurred just before blossoming in all parts of the scape. The ratio of protein to non-protein nitrogen remained constant throughout these stages of growth. Apparently synthesis of protein forms of nitrogen

as well as an accumulation of non-protein forms were occurring during this period.

As the cells began dividing in the scape after blossoming (after embryo development had started), the percentage of nitrogen in the dry matter remained nearly constant. The total amount of nitrogen in the entire scape apparently did not decrease. The amount of nitrogen per cell, however,
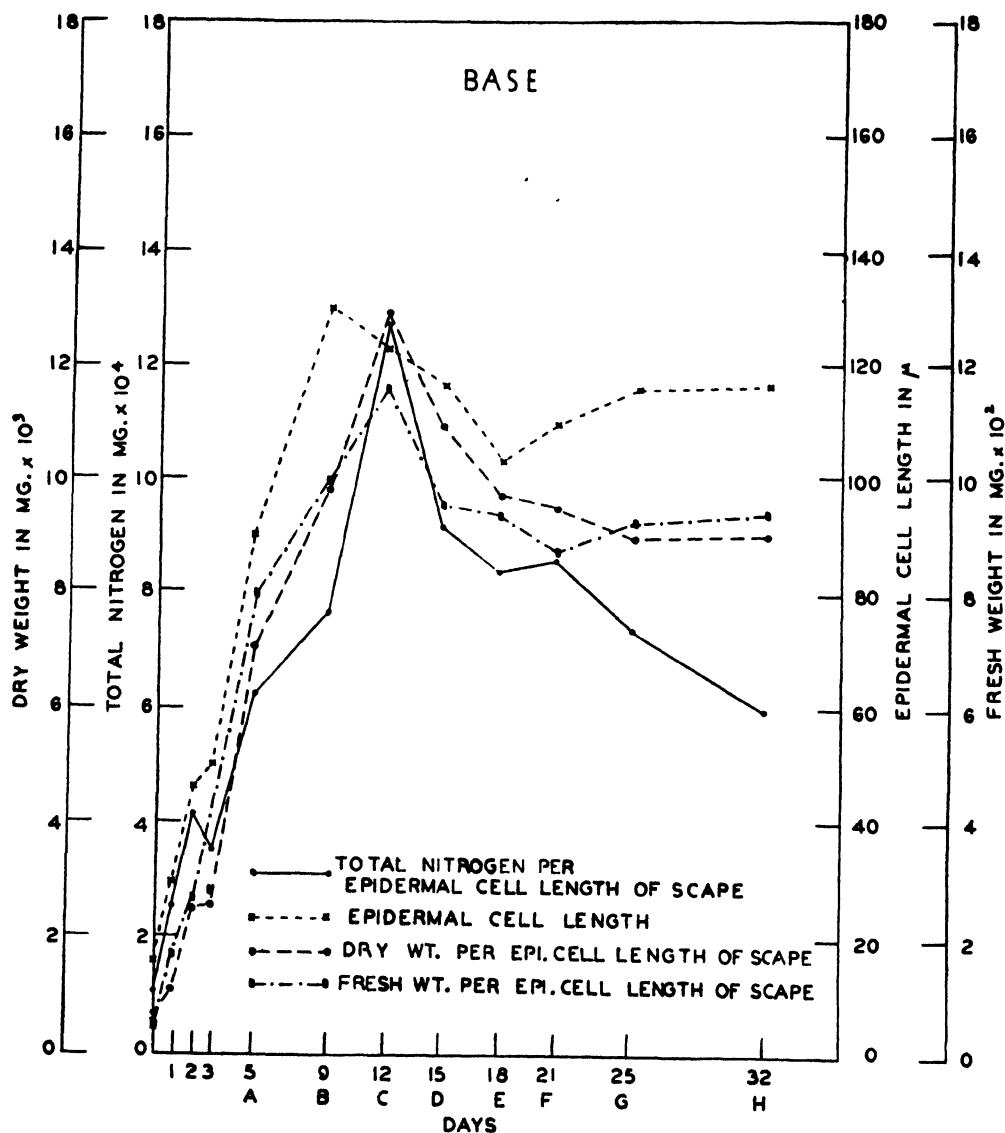
FIG. 6. Total nitrogen, fresh weight, and dry weight, in relation to epidermal cell length at the bottom of the growing dandelion scape.

decreased with division, and as the ratio of protein to non-protein nitrogen still remained the same as in the bud or blossom scape, it appears that synthesis of protein nitrogen during division of the scape cells probably did not occur to any extent at this time.

During the period of rapid cell elongation which preceded and accom-

panied the opening of the seed head (roughly stages "E" to "G") the nitrogen per epidermal cell showed no increase. There was, in fact, some indication of a decrease of nitrogen per cell at this time, especially at the bottom of the scape (fig. 6) which suggests a translocation of nitrogen out of the scape cells. The ratio of protein to non-protein nitrogen which had been constant at all preceding stages, now decreased, indicating that proteins were being digested, and probably that the products of digestion were moving out of the scape into the developing fruit.

The dry weight and fresh weight changes during the growth of the scape are plotted together with the nitrogen and epidermal cell length changes (figs. 4, 5, 6). Dry weight per cell increased greatly during the first period of rapid cell elongation and remained practically constant during the period of slow scape growth after blossoming. During both periods of cell elongation the increase of fresh weight per cell was somewhat greater than the increase of dry weight per cell.

Discussion

In general, epidermal cell length and protein nitrogen, non-protein nitrogen, dry weight, and fresh weight per cell increased and decreased at similar rates until the epidermal cells began to elongate rapidly for the second time, unaccompanied by a corresponding increase of total nitrogen or dry weight, and with only the fresh weight and non-protein nitrogen per cell showing any tendency to increase. The evidence that protein nitrogen formation occurred rapidly during cell elongation at flowering and only to a small extent, if at all, during divisions of cells after flowering was somewhat surprising in view of the widely existing impression that cell division involves great increase of protoplasmic material and that cell elongation involves little or none. The formation of proteinaceous material during cell elongation has been observed to occur in the coleoptile of *Zea mays* (5) and in the hypanthium of *Oenothera acaulis* (3). In studies of growth of broccoli cotyledons evidence has also been found for protoplasm formation during cell enlargement. Cotyledons of seedlings germinated in sand and later transplanted into fertile soil enlarged three to four times after transplantation, whereas the cotyledons of seedlings remaining in sand showed little or no growth. No cell division was involved in this growth, cotyledon and cell enlargement being of the same order. In a typical experiment the cotyledon volume increased 3.71 times, total nitrogen 2.30 times, protein nitrogen 1.74 times, and non-protein nitrogen 2.73 times following transplantation. These data indicate that some substance, possibly available nitrogen, supplied by the more fertile medium was capable of reviving and extending cell enlargement in the absence of cell division in the cotyledons. The rapid rate of protein accumulation in these young, enlarging cells of the broccoli cotyledon is interesting, but a direct relationship between protein synthesis and cell elongation in general is not established, since cell elongation in intact dandelion scapes at the time of seed maturity occurred without increase of

protein nitrogen per cell, and sections of young scapes in sugar and hormone solutions grew rapidly by cell elongation without additional nitrogen. The bud-scapes which elongated in solutions in the absence of an external source of nitrogen, however, had cells which were small and rich in protoplasm as elongation began. If cell enlargement occurs through the stimulation of a basic protoplasmic process which in turn brings about secondary effects (**4, 11**), it may be that synthesis of protoplasm is not the direct means of bringing about cell enlargement, but that a sufficient supply of protoplasm is prerequisite to the initiation of the process. A relationship between protein synthesis and auxin synthesis may also be involved (**1**).

Since elongation may accompany cell division in cells of the dandelion scape, or the two processes may occur independently, it would be of interest to discover which conditions stimulate cells to divide, which stimulate cells to elongate and under what conditions both processes occur more or less simultaneously. The two processes are undoubtedly more closely related and more nearly subject to the same stimulating mechanism than has been generally suspected. GOODWIN and STEPKA (**6**) have shown that the region of most active cell divisions in Phleum roots is also the region of most active cell elongation. Experiments reported in this paper have indicated that chemical reactions and synthesis of protoplasm are found in enlarging as well as dividing cells, and, in fact, that cell division in the dandelion scape does not necessarily involve protein synthesis. It may be that the difference between the mechanism stimulating division and that stimulating enlargement of cells is very slight.

The prominence of protein synthesis in the first period of rapid cell elongation in the dandelion scape and its absence in the second suggest that the stimulus for cell enlargement may be either internal or external to the enlarging cell. If this stimulus be hormonal, it may be postulated either that protein and hormone synthesis within the cell (**1, 2**) stimulates enlargement of the same cell, or that an external supply of hormone from active buds, leaves or fruits above may accomplish the same result. In the broccoli experiments, growth of the entire plant was checked by nutrient deficiencies and cell enlargement in the cotyledons stopped, to be resumed when a supply of soil nutrients made possible the general resumption of protein and presumably of hormone synthesis.

The effect of increasing temperature in accelerating the rate of cell elongation in sections of the scape clearly indicates the presence of limiting chemical reactions either in the wall or in the protoplasm and furnishes additional evidence of the complexity of cell enlargement processes.

Summary

A comparison of organ growth and cell growth was made in dandelion scapes of field plants. Cells were simultaneously dividing and increasing in length until the blossom began to open. At this time scape growth was accelerated and few or no cell divisions occurred. After the blossom closed,

scape growth was very slow and cells were reduced in length through cell division. As the seed head opened, the scape again grew rapidly by cell elongation. During the first rapid elongation the whole scape grew uniformly, although there were probably differences in the relative roles of cell division and elongation in the upper and lower portions, but during the second rapid elongation most of the growth took place at the upper end of the scape.

One-centimeter sections of small bud-scapes elongated rapidly in nonsterile, indoleacetic acid-sucrose solutions, tripling their length in several days under the most favorable conditions. The rate of cell elongation was accelerated by increasing temperatures from 0° to 25° C. The importance of chemical reactions in cell enlargement is indicated.

Total nitrogen as well as protein nitrogen, fresh weight, and dry weight per cell increased during the first rapid growth of the scape. During the second period of rapid elongation, dry weight and protein nitrogen per cell decreased, and fresh weight and non-protein nitrogen per cell increased only slightly. Protein synthesis may or may not accompany cell elongation in scape tissue, and the hypothesis is advanced that cell enlargement may be stimulated either by hormones produced during protein synthesis within the enlarging cell, or by hormones moved from a center of cell activity external to the stimulated cells.

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SOME EFFECTS OF TEMPERATURE AND PHOTOPERIOD ON FLOWER FORMATION AND RUNNER PRODUCTION IN THE STRAWBERRY

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(WITH THREE FIGURES)

The regional and geographical adaptation of strawberry varieties is known to be influenced by day-length and temperature. As DARROW and WALDO (3) have shown, the ordinary varieties of strawberries are short-day plants, initiating flower buds when the light period per day approximates 10 hours or less. According to DARROW (2), temperature exerts a modifying influence on the response of the strawberry to the photoperiod.

In this investigation the response of the strawberry to certain temperature and day-length treatments was studied under controlled environmental conditions. The strawberry is well adapted for use in studies of this kind as it is known to have definite photoperiodic responses which are influenced by temperature. These responses are judged by criteria that are definite and easily measured; namely, the formation of flower clusters when the plant is in the reproductive state and the production of runners when it is in the vegetative condition. In addition the strawberry can be propagated vegetatively by runners, thereby furnishing plant material of the same genetic constitution for experimental treatment.

The equipment used in the present investigation consisted of a set of four environment-control cabinets, described in detail by HARTMANN and McKINNON (5). With these cabinets the temperature could be maintained at desired levels by electric heaters and refrigeration units, both thermostatically controlled. The plants were grown under banks of fluorescent lamps, which maintained a light intensity of about 500 foot-candles at the level of the plants. The day-length conditions could be adjusted at will and were automatically maintained by a time switch.

Methods and results

To test the response of the Missionary strawberry to short and long light periods under the controlled environmental conditions used in this investigation a preliminary experiment was carried out. Twenty plants of this variety, which had been growing in long days and were definitely in a vegetative state, were placed in the cabinets on July 9, 1942. Ten plants were maintained under "short-days" (10 hours) and ten were put under "long days" (15 hours). They were grown in sandy loam in gallon cans. Flower and runner counts were made at intervals from July 23 to October 15. The results are given in table I and definitely show this strawberry variety to be

vegetative under a 15-hour photoperiod and reproductive with a 10-hour photoperiod.

EXPERIMENT I. THE EFFECT OF CONSTANT TEMPERATURE IN COMPARISON WITH FLUCTUATING TEMPERATURE ON THE PHOTOPERIODIC RESPONSE OF THE STRAWBERRY

It was desired to conduct experiments on photoperiodic response with plants maintained under constant temperature conditions. Under natural conditions plants are, of course, subjected to a daily fluctuation of temperature in addition to the daily alternation of light and darkness. That is, the temperature is higher during the light period than during the dark period.

TABLE I

THE EFFECT OF 10 AND 15 HOUR PHOTOPERIODS AT A CONSTANT TEMPERATURE OF 70° F. ON VEGETATIVE AND REPRODUCTIVE GROWTH OF THE MISSIONARY STRAWBERRY. (PLANTS PLACED UNDER TEST CONDITIONS JULY 9, 1942)

DATE	NUMBER OF RUNNERS REMOVED FROM 10 PLANTS		NUMBER OF FLOWERS PRODUCED BY 10 PLANTS	
	10 HOUR DAYS	15 HOUR DAYS	10 HOUR DAYS	15-HOUR DAYS
July 23	4	18	0	0
August 6	0	10	0	0
August 20	0	24	0	0
September 3	0	24	0	0
September 17	0	17	0	0
October 1	0	23	13	0
October 6	0		24	0
October 9	0		32	0
October 13	0		29	0
October 15	0	20	41	0
Total	4	136		

This experiment was designed to determine whether this daily temperature fluctuation is a factor influencing the production of flowers in the strawberry. Two conditions were maintained: (1) short days (10 hours) with the temperature constant at 70° F.; (2) short days (10 hours) with a temperature of 80° F. during the light period and 60° F. during the dark period. Thirty-six plants of the Missionary variety, which had been maintained under long days and were in a vegetative state, were placed under each of these conditions January 11, 1946. Flower counts were started March 19. In recording the results (flower formation), the plants were considered to be in the reproductive state when there was the first macroscopic evidence of flower-bud formation. This condition is determined by the appearance of a cluster of small flower buds in the crown of the plant. Later this flower cluster emerges on the end of a flower stalk or peduncle. Fruit set was recorded when there was the first indication that the fleshy receptacle was swelling. The fruit was considered ripe when it attained a solid red color. The results of this test are given in table II.

All plants under both conditions formed flowers and set fruits that ripened. This occurred earlier under fluctuating than under constant temperature. The difference in time of flower differentiation in the two groups is small. Nearly half the plants under the fluctuating temperature had, however, set fruits before any in the other group had done so; some ripe fruits occurred three weeks earlier at fluctuating than at constant temperature.

EXPERIMENT II. THE PHOTOPERIODIC RESPONSE OF THE STRAWBERRY UNDER ARTIFICIAL CONDITIONS OF (1) LONG DAYS WITH LONG NIGHTS AND (2) SHORT DAYS WITH SHORT NIGHTS

It was shown in the preliminary test and in Experiment I that the strawberry becomes reproductive with a light period of 10 hours and a dark period

TABLE II

COMPARISON OF FLOWER FORMATION, FRUIT SETTING, AND FRUIT-RIPENING OF MISSIONARY STRAWBERRY PLANTS GROWN UNDER CONDITIONS OF CONSTANT TEMPERATURE AND FLUCTUATING TEMPERATURE

DATE	NUMBER OF PLANTS FORMING FLOWERS (36 TOTAL)		NUMBER OF PLANTS SETTING FRUITS (36 TOTAL)		NUMBER OF RIPE FRUITS PICKED	
	60° TO 80° F.	70° F.	60° TO 80° F.	70° F.	60° TO 80° F.	70° F.
March 19	10	7	0	0	0	0
March 27	25	21	6	0	0	0
April 1	28	21	6	0	0	0
April 9	31	30	14	0	0	0
April 12	33	30	19	8	0	0
April 18	35	32	25	22	7	0
April 24	36	34	30	22	3	0
April 28	36	35	31	25	17	0
May 3	36	35	34	32	16	13
May 6	36	36	35	34	23	26
May 10	36	36	36	36	23	30

of 14 hours per day at a constant temperature of 70° F. It is possible that the initiation of the reproductive condition may be due to the short (10-hour) light period, the long (14-hour) dark period, or the combination of the two.

This experiment was arranged to study the effect of the short light period and the long dark period on flower formation in the strawberry. Two of the four cabinets had the light-controlling time clock set to provide a 20-hour cycle: 10 hours of light followed by 10 hours of darkness, etc. This gave the short light period, but not the long dark period. The time clock controlling the lights in the remaining two cabinets were arranged to provide a 28-hour cycle; that is, 14 hours of light followed by 14 hours of darkness, etc. This resulted in the long dark period, but not the short light period.

Forty-two plants were put in the cabinets under each set of conditions

on May 13, 1946, having been maintained previously under long-day conditions. The temperature was held constant at 70° F. Vegetative response was measured by counts of runners produced, and reproductive response by flower clusters formed. The results of these measurements appear in table III.

The plants in both series continued to produce runners from the time they were placed under the test conditions on May 13 until about June 14, a period of 4½ weeks. This situation was due to runner initials which were formed while the plants were under long days previous to being placed in the conditions of the test. From June 14 to the conclusion of the test there

TABLE III

THE EFFECT OF 20-HOUR AND 28-HOUR PHOTOPERIODIC CYCLES ON RUNNER PRODUCTION AND
FLOWER FORMATION IN THE MISSIONARY STRAWBERRY. (PLANTS
PLACED UNDER TEST CONDITIONS MAY 13, 1946)

DATE	VEGETATIVE RESPONSE (RUNNERS REMOVED FROM 42 PLANTS)		REPRODUCTIVE RESPONSE (NUMBER OF PLANTS WITH FLOWER CLUSTERS; 42 PLANTS TOTAL)	
	28-HOUR CYCLE	20-HOUR CYCLE	28-HOUR CYCLE	20-HOUR CYCLE
May 23	25	32	0	0
May 29	36	26	0	0
June 3	27	12	0	0
June 6	11	17	0	1
June 12	6	1	1	2
June 14	0	2	1	2
June 22	2	2	2	2
July 1	2	0	2	2
July 13	3	0	6	2
July 16	8	0	6	4
July 23	19	0	17	21
July 29	24	0	22	26
August 7	44	0	23	41

was a striking difference in the production of runners. The plants maintained under conditions of short light periods and short dark periods (20-hour cycle) ceased runner production altogether, whereas the plants under long light and long dark periods (28-hour cycle) returned to runner formation. The plants under the last-named condition had a temporary decline in formation of runner initials, probably occurring when they were changed from the normal 24-hour cycle to the artificial 28-hour cycle.

As for reproductive response, the plants in the two groups behaved similarly in their production of flowers, except for the reading made on August 7. At this time, in the 20-hour group, 41 out of 42 plants had flowers, whereas in the 28-hour group only a little over half the plants had flowers appearing. Plants grown with long light and long dark periods (28-hour cycle) produced runners and flowers simultaneously. Whenever flower clusters were formed under any of the conditions, they continued to develop, and set fruit.

EXPERIMENT III. PHOTOPERIODIC INDUCTION IN THE STRAWBERRY

In the occurrence of photoperiodic induction, plants can be placed under those conditions of daily light duration which are favorable for initiation of floral primordia and, if kept there sufficiently long, will continue flower development even though they are subsequently moved to photoperiodic conditions unfavorable for flower formation.

In this experiment strawberry plants of the Missionary variety were obtained which originated as runner plants from a field planting that had been under long-day (midsummer) light conditions. They were potted and

TABLE IV

VEGETATIVE AND REPRODUCTIVE RESPONSE OF PLANTS HELD FOR DIFFERENT INTERVALS OF SHORT-DAY (10 HOURS) TREATMENTS FOLLOWED BY SUBSEQUENT EXPOSURE TO LONG-DAYS (15 HOURS)

DATE	NUMBER OF DAYS EXPOSED TO SHORT PHOTOPERIODS			
	3 DAYS*	7 DAYS†	21 DAYS‡	38 DAYS§
VEGETATIVE RESPONSE (RUNNERS REMOVED FROM 10 PLANTS)				
August 20	7	3	6	4
August 27	11	9	5	2
September 6	13	10	3	2
September 16	11	10	0	0
September 23	10	11	6	0
September 30	11	6	4	0
October 7	6	3	0	0
October 14	7	5	2	0
October 21	9	8	2	8
October 26	7	5	4	2
REPRODUCTIVE RESPONSE (NUMBER OF PLANTS WITH FLOWER CLUSTERS; 10 PLANTS TOTAL)				
September 30	0	4	9	6
October 7	0	5	9	10
October 14	0	5	10	10
October 21	0	5	10	10
October 26	0	5	10	10

* Moved to long-day conditions August 13.
† Moved to long-day conditions August 17.
‡ Moved to long-day conditions August 31.
§ Moved to long-day conditions September 17.

placed in the cabinets on August 10, 1946, under short-day conditions (10 hours light and 14 hours darkness per 24 hours). The forty plants used were divided into four groups of ten plants each. Each group remained in the short-day cabinets for a different period of time, then was removed to long days (15 hours of light and 9 hours of darkness per 24 hours). The short-day time periods for the four groups were as follows: (1), 3 days; (2), 7 days; (3), 21 days; (4), 38 days. Results of this experiment were obtained by making counts of runner production and flower formation at intervals. These counts are given in table IV. Throughout this experiment the plants were held at a constant temperature of 70° F.

Floral primordia were initiated when the plants were exposed to 7 photoperiodic cycles consisting of 10 hours of light and 14 hours of darkness per 24 hours. These primordia continued to develop even though the plants were subsequently placed under long-day conditions (15 hours light and 9 hours darkness per 24 hours). Exposure to 3 short-day cycles failed to result in initiation of floral primordia. This fact would indicate that the critical number of short-day cycles required for initiating floral primordia in the Missionary strawberry lies between 3 and 8. Exposures to longer "induction periods" (21 days and 38 days) also resulted in initiation and development of flower clusters. An inverse relation was apparent between runner production and flower formation. Those plants initiating and producing the greatest numbers of flower clusters developed the fewest runners.

EXPERIMENT IV. TRANSLOCATION OF A FLOWER-FORMING STIMULUS IN THE STRAWBERRY

Theories have been proposed (1, 6, 7, 8) that the floral primordia in plants may be initiated by a flower-forming stimulus which is manufactured in the leaves when they are under the proper environmental conditions. This stimulus is presumably translocated to the growing points of the plant, where it results in the differentiation of floral parts. With the equipment available for this investigation, this theory in regard to the strawberry could be tested.

Two types of experiments were performed: one with the leaves, the other with runners. In the test with leaves the plant itself (crown and roots) was maintained under long-day conditions, but the leaves (one or more) were placed in short days. This arrangement was made by cutting an opening in the partition between the short-day and long-day cabinets and letting the leaf petioles extend through the opening. The openings were light-proofed by packing around the petioles with felt. The Missionary variety was used, and the temperature was held constant at 70° F.

HAMNER and BONNER (4) have shown that fully expanded leaves of the short-day plant Xanthium, exposed to long photoperiods, inhibited the transmission of the stimulus through the stem to which the leaves were attached. STOUT (9), working with the sugar beet, a long-day plant, believed that the substance conducive to reproductive development might be translocated with the carbohydrates.

In view of such evidence this test was subdivided into three groups: (1), all the leaves of each plant were maintained in short days; only the roots, crown, and part of the petioles were contained in the long-day cabinets; (2), fifty % of the leaves were held under short-day conditions, while the remaining fifty % were in the long-day cabinets together with the roots and crown of the plant; (3), one leaf only was placed in the short-day cabinet, the remainder of the plant being under long days. Five plants were used in each group and on August 10, 1946, were arranged in the manner described above.

Results were obtained at intervals throughout the test by counts of runner production and flower formation. These results are given in table V.

Plants held in long photoperiods became reproductive when portions of the leaf area were subjected to short days. The number of flower clusters produced was in direct ratio to the percentage of the total leaf area placed under short-day conditions. The formation of runners was in inverse ratio to the percentage of total leaf area held under short-day conditions. Plants with the entire leaf area exposed only to long days formed no flower clusters, but produced more runners than did any other group among the four.

TABLE V

THE EFFECT OF SHORT PHOTOPERIODS ON PORTIONS OF THE LEAF AREA OF PLANTS MAIN-
TAINED UNDER LONG-DAY CONDITIONS IN REGARD TO THEIR VEGETATIVE
AND REPRODUCTIVE RESPONSE. (PLANTS PLACED UNDER
INDICATED CONDITIONS AUGUST 10)

DATE	PORTION OF THE LEAF AREA HELD UNDER SHORT PHOTOPERIODS			
	0 LEAVES (CONTROL)	1 LEAF	50% OF LEAF AREA	100% OF LEAF AREA
VEGETATIVE RESPONSE (RUNNERS REMOVED FROM 5 PLANTS)				
August 20	3	3	2	4
August 27	7	5	2	0
September 6	6	5	3	1
September 16	4	3	0	0
September 23	6	4	0	0
September 30	5	6	1	0
October 7	7	4	1	0
October 14	3	3	1	0
October 21	6	3	0	0
October 26	4	3	0	0
Total	51	39	10	5
REPRODUCTIVE RESPONSE (NUMBER OF PLANTS WITH FLOWER CLUSTERS; 5 PLANTS TOTAL)				
September 23	0	0	0	0
October 7	0	0	0	0
October 14	0	0	1	1
October 21	0	1	2	4
October 26	0	1	2	4

In the second type of test for a flower-forming stimulus in the strawberry, runner plants were used which were attached to the parent plant. The parent plants were secured from a field planting growing under long-day (mid-summer) conditions; each plant selected had a long runner attached. The parent plants were transplanted to gallon cans and placed in the short-day cabinets. The runners were threaded through an opening in the partition into the long-day cabinets. There they were "layered" in soil in pots, where they rooted and grew into plants. Thus, after the beginning of the test on August 10, runner plants were continuously grown under long days, while the parent plants were under short days. Figure 1 shows the arrangement of the parent plant and runner plant in relation to the partition between adjacent cabinets. Half of the runner plants were kept de-
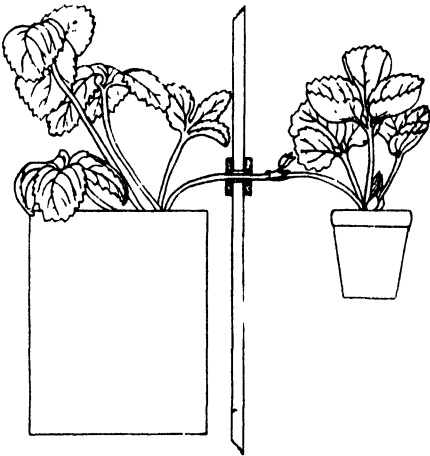
FIG. 1. Method of arranging parent plant and attached runner plant so that each could be maintained under different photoperiodic conditions.

foliated, whereas in the other half the leaves developed normally. By this means a more rapid movement of materials to the defoliated plants from the parent plants seemed likely to occur. The Missionary variety was used in this test, and the temperature was kept constant at 70° F. Counts of runner production and flower formation, taken at intervals, are given in table VI.

TABLE VI

VEGETATIVE AND REPRODUCTIVE RESPONSE OF RUNNER PLANTS GROWING UNDER LONG PHO-
TOPERIODS (15 HOURS) WHEN ATTACHED BY RUNNERS TO PARENT PLANTS
MAINTAINED UNDER SHORT PHOTOPERIODS (10 HOURS).
(TREATMENT STARTED AUGUST 10, 1946)

DATE	RUNNER PLANTS IN LONG DAYS ATTACHED TO PARENT PLANTS IN SHORT DAYS		CONTROL PLANTS GROWING IN	
	DEFOLIATED	NON-DEFOLIATED	LONG DAYS	SHORT DAYS
VEGETATIVE RESPONSE (RUNNERS REMOVED FROM 5 PLANTS)				
August 20	0	0	3	2
August 27	0	0	7	0
September 6	1	0	6	0
September 16	0	1	4	0
September 23	0	7	6	0
September 30	0	2	5	0
October 7	0	1	7	0
October 14	0	2	3	0
October 21	0	0	6	0
October 26	0	0	4	0
Total	1	13	51	2
REPRODUCTIVE RESPONSE (NUMBER OF PLANTS WITH FLOWER CLUSTERS; 5 PLANTS TOTAL)				
September 30	0	1	0	0
October 7	0	2	0	0
October 14	1	2	0	5
October 21	1	3	0	5
October 26	3	3	0	5

The runner plants used were necessarily younger than the long and short-day control plants, which were of the same age as the parent plants.

As shown by the data given in table VI and by the appearance of typical plants in figure 2, runner plants growing under long-day conditions formed flower clusters; provided, however, that they remained attached by runners to plants growing under short-day conditions. Flower clusters formed on those runner plants whose leaves were removed as well as on the non-defoliated plants. The latter were more vigorous, however, and initiated flower clusters sooner, or at least developed more rapidly. This situation might be expected, since these plants would have a greater carbohydrate supply than the plants that depended entirely on carbohydrate movement through the runner from the parent plant. Not all of the runner plants formed flower

SHORT DAYS   LONG DAYS
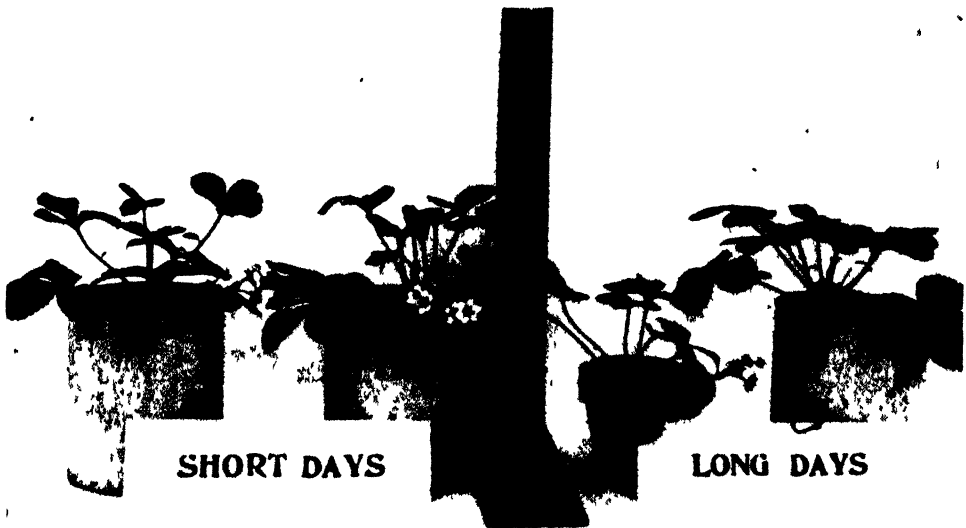
FIG. 2. Appearance of typical plants when grown under short-day and long day conditions. The runner plant (growing in long days) produced flowers because of its attachment to the parent plant (growing in short days).

clusters even though they were attached by runners to plants growing in short days. It was noted that in such plants the runners connecting the two plants turned brown and shrivelled; these runners were probably incapable of translocating food materials or any floral-inducing stimulus.

The plants attached by runners to parent plants growing in short days produced fewer runners than the control plants grown under long days. Allowing leaves to develop on the attached runner plants (growing in long days) increased their vegetative response, as is shown by the increased production of runners in comparison with those attached runner plants that were defoliated.

Discussion

Although flower buds appeared at about the same time under conditions of constant temperature and fluctuating temperature, there was apparently

a constant and consistent hastening of flower and fruit development on plants grown at the fluctuating temperatures used. The plants in the 60°-to-80° F. group had a lower "average temperature" than the plants held constantly at 70°: they were at 80° for 10 hours, at 60° for 14 hours. Nevertheless, the plants in the 60°-to-80° group had a greater developmental rate than those in the 70° group. This condition may be explained by the temperature coefficient of at least 2, characteristic of most plant-cell reactions, which would cause an accelerated metabolism of the plant cells at the 80° F. temperature. For example, if x equals the rate of cell metabolism per hour at a temperature of 70°, the plants held at this temperature would have a relative metabolic rate of 24x in one day. The plants in the 60°-to-80° temperature group would have a metabolic rate of 2x for the 10 hours at 80°, or 20x; they would also have a metabolic rate of 0.5x for 14 hours at 60°, or 7x. This would then total 27x for the 60°-to-80° F. group in comparison with the 24x rate for the 70° group. Thus the plants held under the fluctuating temperature conditions (60° to 80° F.) could be expected to show more rapid developmental characteristics, because of the greater daily metabolic rate.

The rate of photosynthesis would supposedly be the same for the two groups of plants even though the latter were held under different temperatures (70° and 80° F.) during their light periods. As the light intensity used was relatively low (about 500 foot-candles) the "light reaction" was presumably the limiting factor in the photosynthetic process. As this reaction has a temperature coefficient of 1, the different temperatures involved would therefore not influence the photosynthetic rate.

Under field conditions the Missionary strawberry will form runners abundantly during summer with 14 hours of light daily, accompanied, of course, by 10 hours of darkness. Under the conditions used in the second experiment, plants produced runners with a 14-hour daily light period together with a 14-hour dark period, the 14-hour dark period being substituted for the accompanying short (10-hour) dark period found in nature. When a short dark period (10 hours) was used together with a short light period rather than the normal accompanying long light period, no runners were produced. From these facts one may conclude that the long light period appears to be necessary for runner production (vegetative response) rather than the short dark period.

In regard to the reproductive response of the plants used in Experiment II, it is noted that the plants produced flower clusters under both the 28-hour and the 20-hour cycles. Plants grown with long light and long dark periods (28-hour cycle) produced runners and flowers simultaneously.

Four photoperiodic combinations of day and night are possible: (1), long days and short nights; (2), short days and long nights; (3), long days and long nights; and (4), short days and short nights. The first two occur in nature as a 24-hour cycle. Flower formation occurs in the Missionary strawberry under natural conditions only in the second case (short days and long

nights). As table III indicates, flower formation occurred also under the the two artificial conditions: long days with long nights, and short days with short nights. Thus, of the four possible photoperiodic combinations, in only one—long days and short nights—does flower formation fail to occur. If a hormonal mechanism is responsible for flower formation it is possible that a substance may be produced during the light period which must be utilized in a subsequent reaction during the following dark period for flowering to occur. If the dark period is of insufficient duration, in relation to the length of the light period, for complete utilization of the substance, the result may be an accumulation that inhibits flower formation. In this investigation flower formation occurred when the light and dark periods were of equal

FIG. 3. Vegetative and reproductive response of the Missionary strawberry to the four possible combinations of light and dark periods. Treatments 1 and 2 can occur naturally as a 24-hour cycle. Treatments 3 and 4 are artificial.

length, regardless of whether they were long or short. Under natural conditions flower formation in the strawberry takes place, within limits, when the dark period is longer than the light period, but not when the dark period is shorter than the light period. This situation is shown graphically in figure 3.

In the experiment on photoperiodic induction in the strawberry, the first flower clusters appeared at the same date (September 30) in all the three groups that became reproductive. The plants were all placed under the short-day conditions at the same date (August 10). Thus about 50 days were required, after the initial exposure to photoperiodic conditions conducive to development of the reproductive state, before flower clusters became visible. The first microscopic appearance of the initiation of floral

primordia (although not observed here) would occur, of course, some time before the date of macroscopic visibility.

Runners initiated by the previous exposure to long days failed to appear on plants in the 38-day group after September 6. This date was 27 days after these plants were moved (on August 10) from long days (conducive to runner formation) to short days (inhibiting runner formation). Assuming that initiation of runner primordia ceased immediately after the plants were placed in short days, these observations would indicate that about 27 days are required after the start of physiological conditions leading to initiation of runner primordia, before the primordia become macroscopically visible.

Further evidence on the length of time between the beginning of initiation of runner primordia and the first visible evidence of runners is given by the appearance of runners in the 38-day group on October 21. This group of plants was placed in long days on September 17, when, presumably, the physiological conditions leading to initiation of runner primordia were started. Thirty-four days later (October 21), runners were macroscopically visible. The length of time elapsing from beginning of conditions favorable to runner formation and macroscopic visibility was thus established to be 27 days in one case, 34 days in the other. This time interval is for the Missionary variety only; for other varieties a different value would perhaps be found.

The results of the experiment on the translocation of a flower-forming stimulus in the strawberry lend support to certain evidence secured by workers with other plant species; namely, that the leaves, when subjected to the proper photoperiodic conditions, will cause the plant to become reproductive even though the remainder of the plant is held under photoperiodic conditions unfavorable to the initiation of the reproductive state. This observation indicates that the portion of the plant sensitive to the photoperiodic treatment is the leaf. It would seem justifiable to assume that a stimulus is translocated from the leaves to the growing point of the plant to cause initiation of the reproductive state. To explain why more of the plants became reproductive when a higher percentage of the leaf area was held under short days, one may suggest that a larger quantity of the flower-forming stimulus is perhaps produced by the greater number of leaves under short days.

In experiment IV plants were induced to become reproductive even though they were grown under photoperiodic conditions (long days) definitely known to be unfavorable to the development of the reproductive state in the strawberry. This reproductive condition was caused by the attachment of these plants by runners to other plants which were growing under photoperiodic conditions (short-days) favorable to the initiation of the reproductive state and which were themselves producing flowers. These conditions are illustrated in figure 2. The runner plants used in this experiment were undoubtedly dependent upon the parent plants, at least early

in their existence, for food materials. Those runner plants which were kept defoliated were more dependent for materials upon their parent plants than the runner plants whose leaves developed normally. As table VI shows, however, both groups became reproductive, developing flower clusters, although the non-defoliated plants formed flower clusters more rapidly. STOUT, in his work (9) with the sugar beet, concluded that the reproductive-inducing substance may be translocated with the carbohydrates. According to the results of the present investigation, the substance inducing reproduction moved equally well under conditions where different amounts of carbohydrate movement could be expected. The flower-inducing stimulus may have been translocated, however, with the carbohydrate stream very early in the life of the runner plants, when both groups were largely dependent upon their parent plants for food materials.

Generally the production of runners (vegetative state) and the formation of flower clusters (reproductive state) in the strawberry seem to be antagonistic. That is, conditions favoring one tend to inhibit the other. This situation occurred in all cases in this investigation with the exception of the one test in which the plants were grown under the artificial conditions of long days (14 hours) and long nights (14 hours). Here the same plants produced runners and flower clusters simultaneously. This observation would indicate, therefore, that the occurrence of the vegetative state does not necessarily preclude reproductive development. That is, the vegetative and reproductive states may occur simultaneously, at least in the strawberry, provided the environmental conditions are favorable.

Summary

1. Missionary strawberry plants were grown under controlled-environment conditions, with the temperature and day-length regulated, for the purpose of studying the effect of these factors on the vegetative and reproductive responses of this plant.

2. Strawberry plants showed about the same positive reproductive response to short photoperiods whether they were grown at constant temperature or at daily fluctuating temperature.

3. A 20-hour photoperiodic cycle (10 hours light and 10 hours darkness) failed to result in runners. A 28-hour photoperiodic cycle (14 hours light and 14 hours darkness) did cause runner production.

4. Flowers were formed under both the 20- and the 28-hour photoperiodic cycle.

5. Photoperiodic induction was found to occur in the strawberry. For the Missionary variety, exposure to seven short photoperiods resulted in flower formation although the plants were subsequently removed to long days. Exposure to three short photoperiods failed to result in floral development.

6. Flower formation occurred in strawberry plants held under long days provided one or more leaves were subjected to short photoperiods. Runner

plants growing in long days were induced to flower, provided they remained attached by a runner to parent plants growing in short days.

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CAROTENOID PIGMENTS IN TUBERS OF THE KATAHDIN VARIETY OF IRISH POTATO

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(WITH ONE FIGURE)

Introduction

Flesh color in Irish potato varieties ranges from almost pure white to a deep canary yellow. The yellowish tinge of "white-fleshed" varieties is intensified after dehydration, becoming a definite yellow if the tubers have been harvested in an immature state, as is the case with material grown in the south (1, 2).

This residual yellow color, when it occurred, was at one time considered by the Army Quartermaster Corps to be due to a faulty technique in dehydration and as a result such material was graded downward. It was soon recognized, however, that this yellow color was due to the presence of naturally occurring carotenoid pigments. CALDWELL, BRUNSTETTER, CULPEPPER, and EZELL (3) showed that 19 "white-fleshed" varieties, representing fully-matured material, averaged 0.021 mg. of total carotenoid pigments per 100 gm. fresh weight, while 3 normally yellow-fleshed varieties averaged 0.138 mg. per 100 gm. fresh weight. This means that when the total concentration of carotenoid pigments is about 1 p.p.m. on a dry weight basis, the yellow color of the dried product will be slight. When the total pigment concentration is about 7 p.p.m. dry weight, the dried product will have a marked yellow color. One of the findings of the present paper is that in the case of the Katahdin variety, harvested in an immature state, there was still a pronounced yellow color in the dried product when the concentration of carotenoid pigments was 3 p.p.m. dry weight.

This paper reports an intensive study of the carotenoid pigments in three samples of the Katahdin variety, with the purpose of separating and identifying as many of the pigments as possible.

Review of literature

Critical work on the number and nature of carotenoid pigments present in Irish potatoes is very scanty. SCHMID and LANG (13) analyzed yellow internal regions of the Kipfler variety in connection with the so-called "yellow-fleck" disease. From the saponified epiphase fraction they isolated a xanthophyll pigment which was purified by adsorption on a CaCO$_3$ column. Its spectral absorption curve had maxima at 4380 and 4660 A, so that they identified the pigment as "beta xanthophyll." A second pigment, isolated from the hypophase fraction by adsorption on $CaCO_3$, had absorption maxima at 4435 and 4725 A, which as the authors noted, are

characteristic both of violaxanthin and taraxanthin. Incidentally, these absorption maxima are given by ZECHMEISTER and TUZSON (16) for the beta isomer of luteol. A third pigment isolated from the epiphase fraction by adsorption on Al_2O_3 showed broad absorption maxima at 4490 and 4770 A and according to the authors resembled the absorption spectrum of alpha-carotene.

EULER, AHLSTRÖM, HÖGBERG, and TINGSTAM (4) in a nutritional study of the value of the carotenoid pigments from steamed samples of a number of yellow-fleshed varieties of potatoes grown in northern Sweden, separated carotene by chromatographic adsorption on a column of Brockman's aluminium oxide. The total carotenoid pigments ranged from 0.77 to 2.58 p.p.m. fresh weight, with the carotene content varying from 0.066 to 0.10 parts per million.[1]

Methods and materials

Three grades of petroleum ether, referred to as Sk F, Sk B, and Sk C, were purified by stirring successively with concentrated H_2SO_4, acid dichromate solution, NaOH, and four changes of distilled water. After stirring with, then filtering through, silica gel the lots of petroleum ether were distilled over solid KOH. Industrial absolute ethanol was used without purification.

Regarding the chromatographic apparatus, two details of design should be noted: (1) the tube in which the adsorbent was packed had a sintered glass filter at the bottom; (2) the tube was jacketed to provide cooling by continuous circulation of absolute ethanol refrigerated by a mixture of solid carbon dioxide and ethanol. This insured chromatographic analyses at a temperature between $-50°$ and $-60°$ F. The adsorbent suspended in Sk F was packed in the chromatographic column by pressure from a tank of nitrogen. After introduction of the sample, the solvent necessary for the development and elution of the various carotenoid zones was forced through the column by this source of pressure. These zones were caught in separate receivers.

An advantage of a refrigerated column is that it permits the use of Sk F whose low boiling point facilitates evaporation in vacuo at room temperature. Since the force of adsorption increases as the temperature is decreased, adsorbents such as magnesium carbonate, the decahydrate of sodium sulphate, or sucrose, which adsorb so feebly at room temperature as to be quite unsatisfactory, perform excellently at low temperatures such as $-50°$ F. Finally chromatographic analyses at low temperatures retard changes such as formation of stereoisomers, or oxidation of unstable carotenoids.

Absorption data were obtained by a photoelectric spectrophotometer which has a Hilger double monochromator with quartz optics. The slit

[1] The figures referring to the results of EULER and associates as given in reference (3) should be divided by 100.

width generally used was 0.06 mm. giving a spectral isolation of one milli-micron for the mercury line located at 4358.3 A, which was used for the routine calibration of the wave length scale.

The data are expressed in terms of optical density which is equal to the difference between the log incident light (I_0) and the log transmitted light (I). The density is the product of (a) the specific absorption coefficient, (b) the concentration in grams per liter of solution, and (c) the cell length in centimeters.

The first sample consisted of Katahdin potatoes that had been grown in Maine during the season of 1943. Normal maturing of the tubers was retarded by a cold rainy autumn; in fact the vines had to be killed by spraying with chemicals in order to harvest the crop. Accordingly the amount of carotenoid pigments was sufficiently great to impart a strong yellow color to the dried product. Before dehydration, the strips of pota-toes were treated with sulphur dioxide to prevent heat-reddening.

The second lot of Katahdin potatoes was grown in Baton Rouge, Louisi-ana, in the spring of 1944. It was also relatively immature so that the dried product had a yellow color. Undried potatoes (Sample II) were analyzed as well as potatoes treated with sulphur dioxide after steam blanching and before drying (Sample III).

The preparation of the crude carotenoid solutions, essentially similar for the three samples, is described in the following steps:

1. The samples were extracted with absolute ethanol.

SAMPLE	GROWN IN	CONDITION	DRY WEIGHT	GROUND IN BALL MILL WITH ETHANOL
1	Maine	Undried	1303 gm.	as finely ground powder
II	Louisiana	Dried	1000 "	as slices previously extracted with ethanol
III	Louisiana	Dried	1000 "	as Julienne strips

After the undried sample was washed, the skin was removed by vigor-ous rubbing with towels. The sliced sample weighing 8056 gm. was de-hydrated and partially extracted with ethanol at about 10° C. for 6 days. After decanting the extract, the residue was ground under ethanol in a ball mill.

2. The alcoholic extracts were filtered and transferred to Sk F by the addition of excess distilled water, concentrated in vacuo, and washed free of residual ethanol. In the removal of these pigments from the ethanol extract, NaCl was used to break up emulsions and to reduce the solubility of hydroxylated carotenoids in ethanol. Care was taken to remove all the pigments from the ethanol extract.

3. The carotenoids were then partitioned between Sk F and 92% methanol. The upper layer (epiphase) was freed from methanol by wash-ing with water, concentrated in vacuo, and made up to volume. The lower layer (hypophase) was transferred to Sk F by the addition of excess NaCl. At this point also the danger of incomplete extraction of hypophasic pig-ments was recognized and avoided as much as possible. The petroleum

ether phase was then washed with water to remove methanol, concentrated in vacuo, and made up to volume.

Heating in order to concentrate extracts was avoided throughout all operations in order to prevent acceleration of reversible stereoisomeric changes or processes of decomposition.

Experimental results

A 10-ml. aliquot from the hypophase stock solution (50 ml.) of Sample I was chromatographed on a column of confectioners' sugar containing 3% starch. Eight zones were developed and eluted into separate containers. Spectrophotometric readings of the corresponding solutions made at wave lengths of 4000, 4450, and 4700 A amounted to 93, 88, and 102%, respectively, of the readings of the unchromatographed solution measured at the same wave lengths. Here, as throughout this work, development and elution of the various zones were obtained by the successive use of solutions of ethanol in petroleum ether, with a low initial concentration (0.05%) which was increased by small amounts, such as from 0.1 to 0.2%, to an upper concentration determined by the intensity with which the top zone was adsorbed. The judicious use of weak and increasingly concentrated solutions of ethanol in petroleum ether has often been of decided aid in the separation of fractions whose forces of adsorption were nearly alike.

The volume of hypophase stock solution of Sample II was 100 ml. An aliquot (10 ml.) was sent through a column of $MgCO_3$ on top of which was a cap of confectioners' sugar. A fraction containing colorless impurities was readily separated. The remaining pigments were rapidly eluted with 3% ethanol in Sk F, evaporated to dryness, and redissolved in a small volume of Sk F. This solution was then fractionated on successive columns of confectioners' sugar. Twelve zones were developed and collected separately.

However, the separation of the components of the hypophase fraction from Sample III presented difficulties. Adsorption on a column of confectioners' sugar alone was so great that effective development of different zones was impossible. Excellent separation was obtained on a column of Na_2SO_4 alone and mixed in various proportions with confectioners' sugar. Thirteen components were developed by elution but in spite of the fact that the column was cooled to a low temperature, only 54% recovery, measured at 4400 A, was obtained. This loss was largely avoided by the use of $Na_2SO_4 \cdot 10H_2O$, alone and as a diluent of confectioners' sugar. From a 10-ml. aliquot of a 100-ml. stock solution 21 components, most of them minor in amount, were developed and eluted separately and a recovery of 88% measured at 4400 A, was obtained.

For the benefit of workers who may desire to use this last absorbent, a few remarks on technique may be helpful. After preparation of $Na_2SO_4 \cdot 10H_2O$, it was stored under 95% ethanol. In preparation for a run a portion was placed on a Buchner funnel and washed free of ethanol

solution by the use of petroleum ether. The salt was then suspended in Sk F and stirred several minutes in a Waring Blendor. The absence of free water is essential because if present in a refrigerated column it will freeze so that elution becomes excessively slow.

Toward the end of this investigation, preliminary stirring in a Waring Blendor of any adsorbents suspended in Sk F was adopted as a routine procedure, since it greatly facilitated uniform packing of a column, a condition necessary for difficult separations.

In every one of the hypophase fractions of the three samples the first zone to be eluted had essentially the same spectral absorption characteristics. While maxima and minima were lacking in the visible region of the spectrum, there were large and rapid increases in densities as the wave length was decreased to 3400 A, the lower limit of our measurements. The absorption curves for these bottom zones were similar to an absorption curve published by McNicholas (10) for a solution of lutein stored 164 days. These zones contained a relatively large amount of colorless impurities, easily soluble in petroleum ether and difficultly soluble in ethanol. The impurities could be readily removed by lowering the temperature of the ethanol solution in a freezing mixture and then decanting. Experiment showed that they were not responsible for the marked absorption in the near ultraviolet region.

Spectrophotometric data on hypophase sub-fractions of the three samples are given in table I. The solvent for the fractions in Sample I was Sk B; for the fractions in Sample II and III, the solvent was ethanol. The wave lengths of the maxima, minima, and shelves or plateaus are tabulated together with their relative densities (R.D.) based on the densities of the principal maxima. The fractions or zones are numbered according to their relative adsorbability. The smaller the number, the less was the adsorbability of the pigment and the nearer its position to the bottom of the column. In Sample III, a number of chromatographic analyses were made involving different columns; the numbers here denote relative ease of adsorption. Fractions omitted fall into two classes: (a) those that appeared to be mixtures and (b) those with concentrations so low that only a few readings could be obtained.

LUTEIN

Fraction 4, Sample II (with ethanol as a solvent) showed good agreement in details of absorption with the curve published by Zscheile, White, Beadle, and Roach (18) with the exception that the maximum at 4775 A and the minimum at 4625 A were displaced slightly toward the ultraviolet.

Fraction 3, Sample I also was lutein. The absorption curve indicates that in this case stereoisomers of lutein were present. According to Zechmeister and Tuzson (16) lutein, like other carotenoids, can be reversibly isomerized. They found two isomers in approximately equal amounts comprising about 40% of the initial material. Dissolved in ethanol, neolutein

TABLE I

SPECTROPHOTOMETRIC DATA ON CAROTENOID PIGMENTS (HYPOPHASE FRACTIONS)
FROM IRISH POTATO TUBERS

FRACTION	MAXIMA	R.D.*	MINIMA	R.D.	SHELVES	R.D.
Sample I	A		A			A
3	4430–4460	100	4600–4620	71		
	4720–4730	89	4260–4270	69		
	4200–4220	69				
4	4460–4490	100	4330–4350	93	4000–4100	68
	4270	95				
5	4420	100	4580	73	4200–4250	77
	4680–4690	83				
6	4200	100	4050	69		
	4480	94	4350	69		
	3975	76				
7	4480	100	4350	71	4600–4750	42
	4200–4230	97				
8	4260	100	4120	74	4420–4470	63
	4010	93				
Sample II						
4	4460	100	4610	73		
	4750	90	4250–4260	70		
	4230–4240	70				
5	4460	100	4610	71	4200–4250	68
	4720–4740	91				
7	4250	100	4400–4430	81	4000–4100	78
	4500	87				
9	4445	100	4620	72		
	4725	90	4275	70		
	4225	71				
10	4400–4450	100	4600	77		
	4650–4700	82				
12	4260	100	4125	74		
	4025	83				
Sample III						
6	4425–4450	100	4600–4625	77	4200–4250	73
	4700–4750	87				
8	4400–4425	100	4600	76	4180–4250	75
	4680–4700	85			4200–4250	84
11	4420	100			4200–4250	84
					4600–4700	86
14	4450–4480	100	4600–4625	76	4200–4250	70
	4725–4760	90				
16	4400–4500	100	4625	80	4550–4600	83
	4700	91				
17	4425–4475	100			4250–4300	91
					4675–4700	90
18	4400	100	4600	78	4200–4250	80
	4650	81				
19	4450–4500	100	4350	94	4250–4300	96
					4700–4725	70
20	4400–4475	100	4625	80	4250–4300	87
	4700	80				
21	4440–4460	100	4350	92	4625–4700	65
	4200–4220	99				

* Relative density based on the densities of the principal maxima.

A gave absorption maxima at 4710 and 4420 A; the corresponding maxima
for neolutein B occurred at 4720 and 4430 A. The broad absorption maxima
of Fraction 3, Sample I was evidence for the presence of stereoisomers.

Further evidence was the presence of a slight peak at 3300 A, for ZECH-MEISTER and POLGÁR (17) state that lutein in equilibrium with its two isomers shows a maximum (the "cis peak") at 3310 A (hexane).

The concentration of lutein in parts per million dry weight was 0.50 in Sample I, and 0.77 in Sample II.

In the processing of Sample III, the strips of potato tissue were steam-blanched for 7 minutes, then placed immediately in a sulphurous acid solution (0.25%) for 30 seconds. As a result the dried product had a pronounced taste, and the initial alcoholic extract had a pronounced odor of sulphur dioxide. As previously stated, the hypophasic pigments with the exception of the first fraction were adsorbed on a column of confectioners' sugar far more strongly than the hypophasic pigments of the other two samples. This pronounced increase in adsorption may be interpreted as due to an increase in the number of hydroxyl groups in the various carotenoid pigments of this sample, a chemical change which need not necessarily be accompanied by more than slight changes in the absorption spectra. These considerations, along with the presence of a shelf instead of a maximum and a minimum in the region 4200–4260 A, appear to rule out Fraction 6, the first major hypophasic fraction of Sample III, as lutein.

Fraction 5, Sample II, was adsorbed immediately above lutein and its absorption curve was closely similar to lutein. The chief difference was the absence of a maximum and a minimum in the region 4200–4260 A. Consequently, this fraction is tentatively identified as isolutein.

FLAVOXANTHIN

This pigment, it will be recalled, was first isolated by KUHN and BROCK-MANN (9) from *Ranunculus acer* (buttercup). KARRER and JUCKER (5) showed that its molecule has two hydroxyl groups, and that the third O atom is present in an ether-like bond. STRAIN (14) demonstrated its presence in green leaves and showed that it is adsorbed above lutein on a magnesia column. STRAIN's measurements of its absorption curve in ethanol solution located the maxima of the two flavoxanthin isomers at 4220 and 4520 A. Among the seven xanthophylls which STRAIN isolated from green leaves, flavoxanthin alone has absorption maxima at these positions.

According to the absorption data in table I for Fraction 6, Sample I, this fraction was a flavoxanthin. Fraction 7, Sample II, does not check well with STRAIN's data, and it is probable that this fraction contained impurities which rechromatographing would have removed. A third fraction, 21 from Sample III, also possessed maxima whose positions approximated those of flavoxanthin. However, in view of its relative adsorbability and the lack of agreement in details with the spectral adsorption curve of flavoxanthin, it is improbable that this fraction contained flavoxanthin.

AUROXANTHIN

This pigment, isolated and described by KARRER and RUTSCHMANN (7, 8), is distinguished by sharp absorption maxima of equal intensity

located at 4030 and 4280 A. Absorption maxima of lesser intensities are located at 3820 and 2940 A.

The absorption data for Fraction 8, Sample I, and Fraction 12, Sample II, had very sharp absorption maxima which checked closely with the two principal absorption peaks of auroxanthin. As in the case of auroxanthin, inflections between 4400 and 4500 A were present. No examination of the absorption was made in the region below 3500 A. In our preparation there were shelves instead of peaks in the region 3800–3900 A. Furthermore, the densities at 4280 and 4030 A were unequal. Further work on a more highly purified preparation would be necessary to establish the identity between this pigment from potato tubers and auroxanthin.

The remainder of the hypophasic pigments listed in table I could not be identified.

EPIPHASE FRACTIONS

The epiphase portion of Sample I was dissolved in Sk F and adjusted to a volume of 50 ml. For chromatographic analysis, an aliquot of 5 ml. was poured on a column of $MgCO_3$ on top of which was a layer of confectioners' sugar. Elution with Sk F alone separated the yellow bottom zone which was the main fraction. Six other zones were developed and separated by elution with solutions of ethanol in Sk F, starting with a 0.1% solution and going by steps to a 3% solution. The third zone from the bottom was further fractionated into a blue zone (which later in the course of elution changed to a green color) with a yellow zone immediately above it. The fourth zone from the bottom was green in color. On top of the layer of sugar there was a blue zone which could not be eluted with a 3% ethanol solution. These interesting zones were not examined spectrophotometrically.

The bottom zone (fraction 1) was rechromatographed on the same type of column. A major bottom zone (fraction 1–1) with two yellow zones adsorbed above it were separated. Absorption curves of these upper zones showed no maxima or minima; both fractions showed a continuous increase in density with sharp increases in rate at 3800–3700 A. The curves were similar to the absorption curves of the first zones eluted from the hypophase fractions.

In the course of this work on the epiphase solutions, commercial beta-carotene was purified by several recrystallizations from Sk F by additions of ethanol and its absorption spectrum was measured. The data will be given here for comparison with the carotene fractions listed in table II: 4525 A, density 100; 4805 A, 87.90; 4700 A, 81.0; 4300 A, density 70.7.

As shown in table II, Fraction 1–1 from Sample I checked fairly well with beta-carotene as regards relative densities; though, as might be expected from unrecrystallized material, there was a displacement of the minimum and the principal maximum toward the ultraviolet. At wave lengths below 400 A the density increased to 3000 A, parallel to that of the other two fractions. The pigment was unstable, for the densities of the principal maxima changed during measurement. This might have been predicted for

(a) the sample had been ground to a fine powder and (b) the original ethanol extract had been stored for about 3 weeks'at − 40° C.

The concentration of Fraction 1–1, calculated as carotene, was 0.38 p.p.m. dry weight.

The epiphase solution of Sample II also was a complex mixture of carotenoid pigments. An aliquot of 5 ml. from a stock solution of 50 ml. was chromatographed on a column of $MgCO_3$. At least nine zones were developed, six of which were obtained separately by elution. The third zone from the bottom was a composite of an orange zone, two yellow zones, and a greenish zone. The sum of the densities measured at 4500 A was 94.6% of the unchromatographed epiphase solution, evidence of minor loss only.

As in the case of Sample I, the bottom zone was the main fraction; measured at 4500 A its density was 42% of that of the total carotenoids in the epiphase solution and 9% of the total carotenoids in both hypophase and epiphase solutions. Calculated as carotene, this fraction was present in a concentration of 0.31 p.p.m., dry weight.

Referring to the spectrophotometric details given under 1, Sample II, in table II, the broad absorption maximum at 4485–4520 A, the shift in position of maxima and minima toward the ultraviolet and the presence of a shelf in the curve at 4250–4300 A indicate that this fraction was a mixture of stereoisomers. The relative densities checked with those of all-trans-beta-carotene with the exception that the curve in the region of 4300 A was about 10% too high.

Fraction 2, adsorbed immediately above fraction 1, was particularly interesting since the relatively high density from 4270 to 4330 A indicates a carotenoid component with an absorption maximum in this region. This point will be reexamined later.

Analyses of the absorption curves of the other zones in Sample II indicated that they too were mixtures, a peculiarity of the third (composite) zone from the bottom being the presence of a pronounced absorption peak at 4120 A.

This run confirmed the results of the analysis of the epiphase fraction of Sample I in showing that a column of $MgCO_3$ even when cooled below − 50° C. was not efficient in separating epiphasic pigments, at least in a single chromatography. Very probably repeated chromatography would have given a better separation. The results clearly show, however, that in the absence of factors such as treatment with SO_2 and drying, a complex mixture of pigments is obtained not only in the hypophase, but also in the epiphase fraction.

When a 10-ml. aliquot of the epiphase fraction of Sample III (100 ml. Sk F) was chromatographed on $MgCO_3$ spectrophotometric measurements of the bottom (main) fraction (table II, Sample III; Run 1–a) showed at once that the relative density at 4275 A was much higher than was the case in the other two samples. This high ratio was present also in the case of the other three upper zones. Rechromatographing this first fraction on a

column with a bottom layer of confectioners' sugar, a main layer of Ca(OH)$_2$, and a top layer of MgCO$_3$, did not change essentially the relative density at 4275 A. Calculated in terms of carotene, this first fraction was present in a concentration of 0.61 mg. per kg. dry weight, almost twice the values obtained for the "carotene" fractions of the first two samples, evidence that it was impure. This bottom zone had a density at 4400 A that was 55% of the total epiphasic density.

The separation was repeated using a 30-ml. aliquot and chromatographing twice on columns of MgCO$_3$, a middle layer of equal parts of MgCO$_3$ and Ca(OH)$_2$, then a bottom layer of confectioners' sugar. Five zones were separated. The ratios of the density values at 4300 and 4500 A were 75 and 238% for the bottom and top zones respectively, showing definitely that both the main fraction in the previous run and the zones adsorbed above it had been contaminated with a pigment absorbing strongly near 4300 A.

The top zone was then chromatographed three times on a column of 2 parts of Ca(OH)$_2$ to 1 part of MgCO$_3$. The final product was characterized by a density at 4300 A that was 626% of the density at 4500 A. Detailed spectrophotometric analysis of this fraction dissolved in ethanol showed the characteristics listed in table II, Sample III, Run 1–b, Fraction 1–a–5–c–2.

In spite of repeated chromatographic analyses, this yellow fraction contained a lower pink zone in relatively small amount which could not be eluted separately. Apparently it did not interfere with the absorption of the yellow zone as the peaks and minima of the latter were very sharp, and corresponded closely with values for a carotenoid given the provisional name of ξ-carotene by STRAIN and MANNING (15). This carotene has been found in carrots, yellow corn, and in certain strains of tomatoes. A review of the literature has been given recently by NASH and ZSCHEILE (11) who have measured its absorption spectrum and present specific absorption coefficients. They state that ξ-carotene exhibits typical isomerisation phenomena and suggest that it may occur in nature in a wide variety of sources. Recently PORTER, NASH, ZSCHEILE, and QUACKENBUSH (12) have found that ξ-carotene is biologically inactive as provitamin A.

To check on the carotene nature of sub-fraction 1–a–5–c–2 (ξ-carotene) it was dissolved in Sk F and shaken for one hour at room temperature with 20 ml. of a saturated solution of KOH in methanol. Subsequent partition by shaking with methanol showed that no pigment transferred to the methanol layer, and that therefore ξ-carotene is not an esterified carotenol. It was then transferred to ethanol, made up to volume, and remeasured after a month's storage at −4° C. A marked general decrease in density was noted. A slight maximum at 3550–3600 A and a slight minimum at 3600 A were observed.

The presence of ξ-carotene was confirmed by a second preparation (Run 2). A subsample of III weighing 334 gm. was extracted by grinding overnight in the ball mill in the presence of two grams of CaCO$_3$ using specially purified absolute ethanol. An aliquot of the filtrate equivalent to 230 gm.

of sample was transferred to Sk F, concentrated to a volume of one liter, then shaken twice in two 500-ml. portions with successive charges of 25 ml. of methanol saturated with KOH. The pigments were partitioned in the usual manner between 92% methanol and Sk F and the two fractions obtained were then transferred to Sk B.

Twenty-five ml. of the epiphase fraction (100 ml.) were chromatographed on a column composed of 40% of $Ca(OH)_2$ and 60% of $MgCO_3$. This separation was unsuccessful; so the pigments were eluted with 4%
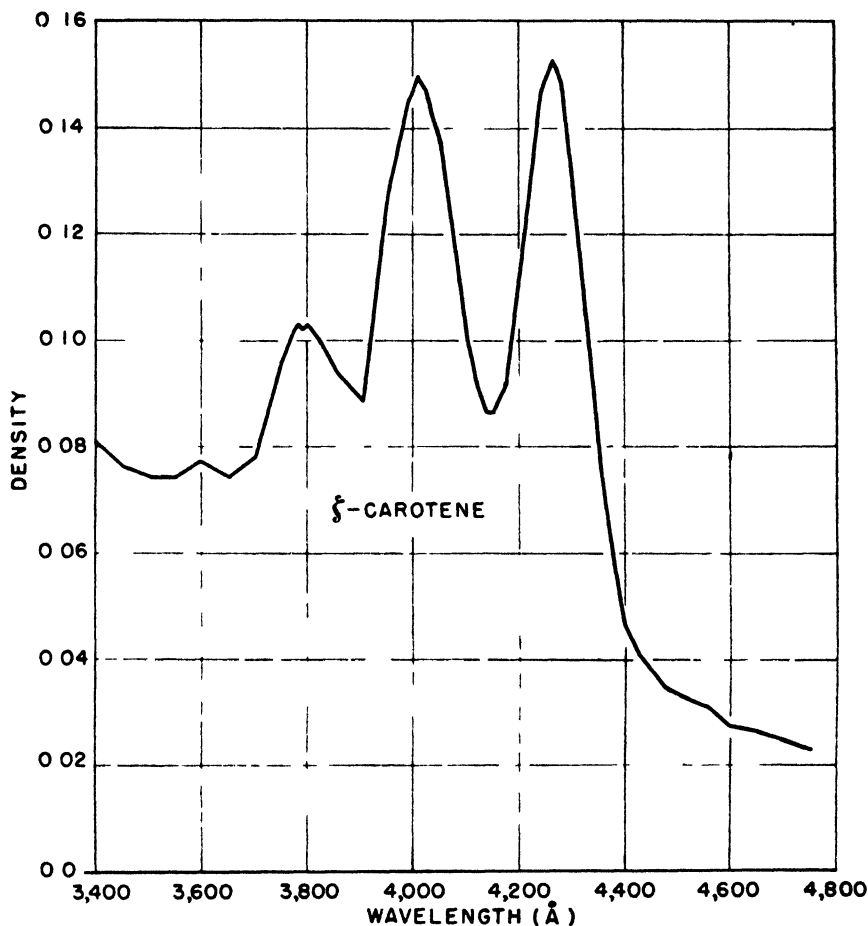
FIG. 1. Absorption spectrum of ζ carotene, separated from crude carotene of epiphase solution, from tubers of Katahdin variety of Irish potatoes.

ethanol in Sk F, evaporated to dryness in vacuo, and redissolved in Sk B. The second column was composed of $MgCO_3$ with a top layer of confectioners' sugar. The bottom zone obtained from this column was rechromatographed on a column of 20% $Ca(OH)_2$ and 80% $MgCO_3$. A yellow bottom zone was eluted with Sk F alone (see table II, Run 2, 2-a; this pigment could not be identified). The zone above it was composed of two sub-fractions (the lower, pink in color; the upper, yellow) which could not be separated sufficiently to be collected separately. The next zone was minor in

amount. At the top of the column there was a yellow zone which was eluted with 0.5% ethanol in Sk F and collected separately. The details of the absorption curve (see table II, Sample III, Run 2–d; compare with 1–a–5–c–2) checked with those of the previous run and showed moreover a slight peak at 3600 A. This curve is reproduced in figure 1. In this run the amount of ξ-carotene obtained was 134% of the amount obtained in the

TABLE II

SPECTROPHOTOMETRIC DATA ON CAROTENOID PIGMENTS (EPIPHASE FRACTIONS)
FROM IRISH POTATO TUBERS

FRACTION	MAXIMA	R.D.	MIMIMA	R.D.	SHELVES	R.D.
Sample I‡	*A*		*A*			
1–1	4500	100	4650	79		
	4800	90				
Sample II‡						
1	4485–4520	100	4660	82	4250–4300	78
	4760	89				
2	4445	100	4630	76	4270–4330	89
	4720	81				
	4200	90				
Sample III*						
Run 1–a 1	4500	100	4687	84		
	4775	86	4350	85		
	4275	94				
Run 1–b: 1–a–1–a	4450	100	4600	77	4200–4500	73
	4760	89*				
1–a–1–b	4500	100	4725–4750	84		
	4725	85				
1–a–3–4–b–3	4475	100	4650	82	4250	73
	4725	86				
1–a–5a	4500	100	4700	82		
	4750	85	4340	83		
	4250–4275	86				
1–a–5–c–2	4240	100	4150	53		
	4000	99	3880	56		
	3800	69				
Run 2: 2–a	4525	100	4700	85	4200	70†
	4775	86				
2–b	4500	100	4700	83	4300–4350	80
	4750	86				
2–d	4260	100	4150	56		
	4010	98	3900	58		
	3800	67	3650	48		
	3600	50				

* Estimated by extrapolation.
† Inflection.
‡ Solvents: Sample I, Sk B; Samples II and III, ethanol.

previous preparation. The density at 4250 A was 12.5% of the entire epiphase fraction. Using the value of 226 for the specific absorption coefficient of ξ-carotene at 4260 A (NASH and ZSCHEILE), the concentration of ξ-carotene was calculated to be 0.11 p.p.m. dry weight.

Returning now to the description of the remaining four zones of Run 1–b, the bottom zone, 1–a–1, was rechromatographed on a column whose top and bottom layers were confectioners' sugar and whose main part con-

sisted of a mixture of 2 parts of $CaOH_2$ and one part of $MgCO_3$. Two sub-fractions, 1–a–1–a and 1–a–1–b, were separated. It is apparent from the spectrophotometric data in Table II that neither of them was trans-beta-carotene and that probably they consisted in whole or in part of cis-trans stereoisomers. The ratio of densities at 4300, 4500 and 4800 A for the two fractions above the bottom zone checked with the corresponding ratios for pure trans-beta-carotene. Calculating their sum as carotene the concentration was 0.19 p.p.m. dry weight, about one-third the concentration of the crude carotene fraction.

Unfortunately complete curves were not obtained for these fractions or for the fourth zone from the bottom. Instead the third and fourth zones from the bottom of the column were combined and rechromatographed on a column consisting of a sugar base, a middle layer of $Ca(OH)_2$ and one part of $MgCO_3$. Three zones were obtained. Data on the top zone are listed in table II under 1–a–3–4–b–3. This fraction dissolved in ethanol was stored at $-4°$ C. for one week and then remeasured. No displacement of absorption peaks had occurred, and the density was only slightly less. This fraction corresponded closely as regards location of peaks and relative densities to neo-beta-carotene-U as described by ZECHMEISTER and POLGÁR (17).

As noted above, the top zone was chromatographed three times on columns consisting of 2 parts of $Ca(OH)_2$ and 1 part of $MgCO_3$ in the separation and purification of ξ-carotene. A fraction obtained after the first chromatographic separation was further resolved and studied in detail. Pertinent data are given in table II under fraction 1–a–5–a. The absorption curve indicated that an unidentified cis type of carotenoid was present.

Discussion

In this work, in spite of the fact that the total amount of carotenoids available for investigation was only about 3 mg., at least a dozen different zones representing different carotenoid pigments were developed on chromatographic columns though not all these could be separated in a form suitable for analysis.

Among them perhaps the most interesting were the two pigments with sharp major absorption peaks around 4000 and 4250 A. One of these was in the hypophase portion and was located at the top of columns of confectioners' sugar. As already noted, the location of the two major absorption peaks agrees fairly closely with those reported by KARRER and RUTSCHMANN (7) for auroxanthin. Their method of separation emphasizes the tenacity with which this pigment is adsorbed, for they employed a column of zinc carbonate, a mild adsorbent, and used for elution a mixture of 1 part of methanol to 2 parts of ether. Their source of material was the flower, *Viola tricolor*.

From KARRER's laboratory a series of recent reports have clarified the relation between major carotenoids and their oxidation products. In gen-

eral, a carotenoid lacking oxygen or containing oxygen only in the form of hydroxyl groups is treated with monoperphthalic acid. This results in an epoxy compound, the oxygen introduced bridging two adjacent carbon atoms in the β-ionone ring. These epoxy derivatives are very sensitive to the action of acids. For example, the minute amount of HCl in "aged" chloroform will rapidly transform dissolved epoxy carotenoids to furanoid oxide isomers. As a result marked displacements of the absorption maxima toward the ultraviolet occur. Specifically KARRER and JUCKER (5) found that lutein epoxide isomerized to form two carotenoids which were identical with flavoxanthin and chrysanthemaxanthin, each containing two –OH groups with the third oxygen atom in an ether-like union. Zeaxanthol has two cyclohexenyl rings and so forms a mono-epoxide and a di-epoxide which were found to be identical with antheraxanthin and violaxanthin respectively. Treated with acid, antheraxanthin isomerized to mutatoxanthin, while violaxanthin isomerized to auroxanthin. The acid treatment split off oxygen to some extent from violaxanthin so that mutatoxanthin and the starting material, zeaxanthin, were also present.

They further found by the same technique (6) that β-carotene also forms a mono-epoxide and a di-epoxide. Treated with acid the former isomerized to mutatochrome and the latter to aurochrome.

Thus from known carotenoids easily obtained in relatively large amounts, other carotenoids, most of them previously isolated from natural sources in small amounts, were prepared by in vitro chemical reactions. KARRER and JUCKER believe that the in vitro method of preparation essentially parallels the processes in the plant which produce these less abundant carotenoids.

It follows then that the detection of auroxanthin in Sample II (undried) implies the presence of violaxanthin and zeaxanthin. These two carotenoids may have been among the fractions studied, but they could not be detected with certainty, probably because the impurities or cis stereoisomers present obscured the none too distinctive absorption spectra. The absorption spectrum of auroxanthin is quite characteristic distinguishing it sharply from all other hypophasic carotenoid pigments, so that its presence in Samples I and II seems to be fairly certain.

It is possible that auroxanthin may be widely distributed and that the reason it has not been generally noted is the fact that only a mild adsorbent with an eluent containing a polar solvent serves to separate it. One would expect it to be held in an uppermost zone on a column such as magnesium oxide. In this connection it is of interest that STRAIN (14) in his monograph on leaf xanthophylls records 11 zones separated on columns of magnesia. The topmost three zones, orange-yellow in color, are denoted as mixtures with two absorption maxima, one at 4500 Å and an "indefinite" maximum at a shorter wave length. The authors suggest that one or more auroxanthin-like compounds may be separated by rechromatographing these upper three zones on a column of either confectioners' sugar or on $Na_2SO_4 \cdot 10H_2O$.

The second pigment of this type was ξ-carotene, appearing in the crude carotene fraction of Sample III. Unfortunately, due mainly to the fact that the aliquots taken for chromatographic analyses were too small, its presence in the carotene fractions of the other two samples could not be definitely established. Another factor which very probably was largely responsible for its apparent absence was the fact that in Sample II the epiphase had been stored in ethanol for 20 days. As STRAIN (15) and NASH and ZSCHEILE (11) have observed, ξ-carotene rapidly oxidizes in polar solvents. Unfortunately this fact was not recognized in the present work so that the concentration of ξ-carotene reported for Sample II is very probably too low. Furthermore, Sample I had been ground to a fine powder so that ξ-carotene, if present, would have been exposed to conditions making for maximum oxidation. In the case of Sample III, only a week elapsed from the extraction of the sample to the storage of the epiphase in Sk B.

KARRER and JUCKER (6) state that the absorption spectra of auroxanthin and aurochrome are practically identical. Similarly there is a close agreement between the absorption maximum of auroxanthin and that of ξ-carotene. Aurochrome is adsorbed above β-carotene on a column of $ZnCO_3$. Similarly, ξ-carotene is adsorbed above β-carotene on a suitable column. In view of these facts the authors suggest that ξ-carotene is identical with aurochrome and therefore is derived from β-carotene by oxidation to the di-epoxide followed by isomerisation to the furanoid oxide form thus reducing the number of conjugated double bonds and displacing the positions of the absorption maxima toward the ultraviolet. If the two pigments are identical then "ξ-carotene" like aurochrome should, when its ethereal solution is shaken with 25% HCl, show a stable deep blue color. Whether ξ-carotene does so react with concentrated HCl is unknown at present.

Although the total amount of carotenoid pigments present in the undried sample of Katahdin potatoes (II) was approximately 3 mg. per kg. dry weight, it was sufficient to give the dried product a marked yellow color. It is interesting to note that dehydration of this sample lowered the total carotenoid content only 10%.

The concentration of beta-carotene was about 10% of the total carotenoids present and obviously it was present in such small amounts as to be negligible from a nutritional point of view.

Summary

1. The Katahdin variety of potato, when harvested in a relatively immature condition, contains a complex mixture of carotenoid pigments. The concentration of total pigments was about 3 parts per million, dry weight, a concentration sufficient to impart a yellow color to the dehydrated product.

2. Lutein was found in two of the three samples studied, in concentrations of 0.50 and 0.77 parts per million, dry weight. A pigment corresponding to isolutein was present in one sample.

3. A flavoxanthin was found in one sample and tentatively identified in a second sample.

4. A carotenoid provisionally identified as auroxanthin was present in two samples.

5. An epiphasic pigment with sharp absorption maxima located at 3800, 4000, and 4250 A was separated from one sample. It was identical with ξ-carotene. Its concentration in potatoes was at least 0.11 parts per million, dry weight. It is suggested that ξ-carotene is identical with aurochrome, derived by KARRER and JUCKER from β-carotene.

6. Beta-carotene was detected; its concentration was about 0.3 parts per million, dry weight. In one of the samples, the presence of the stereoisomer, neo-beta-carotene-U was detected.

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THE SUGARS OF THE ROOTS OF *DAUCUS*[1] *CAROTA*

A . G . G A W A D I

It is well known that some reducing substances, resembling true sugars in their behavior, occur almost invariably in plant extracts and may introduce great errors into the estimation of those sugars. Since the soluble sugars commonly present in plants (glucose, fructose, sucrose and maltose) are easily fermented by yeast, this phenomenon suggests itself as a convenient and practicable method for the fractionation of the total reducing substances in any plant extract into fermentable and non-fermentable fractions.

Methods

TRIALS ON THE FERMENTATION OF PURE SUGARS BY ORDINARY BAKER'S YEAST

After trials with various brands of baker's yeast, a commercial brand, known in England by the name "Eureka" was selected as being most suitable.

Considerable work was done to determine optimum conditions for fermentation of pure sugars, with the selected yeast in the shortest time possible, in order to evade the action of bacteria. The conditions dealt with involved the study of the different hydrogen ion concentrations in the yeast media, the amount of yeast necessary for the complete exhaustion of a given amount of a fermentable sugar, the different sugars the selected yeast can digest, the time required for the completion of the fermentation process, and some other technical points.

The following technique was finally adopted: 10 cc. of 10% washed yeast, 2 cc. acetate buffer (pH 4.7), 2 cc. 0.2 M KH_2PO_4 and 25 cc. sugar solution of known strength are mixed in a 50-cc. measuring flask and incubated at 35° C. for three hours. At the expiration of this period the mixture is neutralized to phenol red with N/10 NaOH, 4 cc. alumina cream are added with subsequent shaking of the flask, the volume is then completed up to 50 cc., and the contents filtered dry. A few drops of toluene are added to the clear filtrate which is set aside for estimation.

Very much smaller concentrations of yeast were found adequate for the complete removal of glucose, fructose, and sucrose in the time stated; maltose fermentation, on the other hand, was much slower. Using the standard concentration it was found that of 50 mg. maltose supplied, 90% had been removed in three hours; while practically complete removal in the same period was achieved when 5 cc. of 5% Taka-diastase solution were added to the yeast suspension.

Trials with galactose and the pentose sugars (xylose and arabinose) under the standard conditions showed that of 35 mg. supplied 96%, 98%,

[1] The variety used in this work is known in England by "Early Market."

and 95% of the original amounts of these sugars were recovered, respectively. The small losses are presumably due to the development of bacteria under the conditions of the incubation, since estimations after twenty-four hours incubation gave very much higher losses; a result which makes HEINZE and MURNEEK'S (5) practice of 48 hours incubation and that of RYGG (11) of 18 hours incubation for fractionating yeast fermentable and non-fermentable parts of plant extracts rather questionable.

Any loss of reducing power due to the absorption, by yeast, of some of the sugars under investigation can be neglected, since experiments in which the fresh yeast was precipitated with alumina cream, immediately after mixing with the sugar solutions, gave satisfactory percentage recovery: maltose, 99%; galactose, 100%; arabinose, 100.5%; and xylose, 99%. Glucose, however, under the same treatment gave a recovery of only 96%, a result which is ascribed to its rapid fermentation, for with boiled yeast treated in the same way the recovery was 101%.

Thus the technique adopted ensures the removal of glucose, fructose, and sucrose, leaving galactose and the pentose sugars practically untouched.

APPLICATION OF THE TECHNIQUE ADOPTED FOR THE REMOVAL OF THE FERMENTABLE SUGARS FROM CARROT TISSUE EXTRACT

Time curves for complete removal of fermentable sugars in carrot tissue extract were studied on many occasions. Because of the presence of some non-fermentable reducing substances which are continually attacked by bacteria present in the yeast suspension, no constant value for these substances could be attained at any point on the time curve. For this reason, the method which was finally adopted to ensure the complete digestion of the reducing fermentable sugars in the tissue extract was as follows.

A sample of the extract (about 20 cc.) was treated with yeast under the standard conditions, and to another aliquot sample a known amount of glucose was added; in both cases the reducing value of the clear digest was the same. This implies: firstly, that fermentable sugars in the extract are totally removed; and secondly, that nothing is present in the extract to hamper in any measurable way the action of yeast.

To rule out any possible error which might arise from the application of yeast, a blank fermentation containing all of the reagents with distilled water in place of the experimental extract, was always carried out. It is interesting to note that the titration value of this yeast blank and that of pure distilled water, were always practically the same, especially when the copper method was being employed for the estimation of reducing values.

The amount of yeast used was decreased or increased according to the reducing value of the material to be fermented, and a corresponding increase or decrease was made in the amount of alumina cream used for precipitation.

EXTRACTION AND CLARIFICATION

For the extraction of carbohydrates from carrot root tissue, the 80%-alcohol technique was employed. Extraction was carried out in a simplified

soxhlet-like apparatus until no color appeared in the drippings. This was considered the end point of extraction, according to previous work done in this laboratory. The simpler carbohydrates, such as the hexoses and the disaccharides, were sought in the alcohol extract, the more complex forms in the alcohol insoluble residue. From the extract, the alcohol was removed by distillation under reduced pressure at 50°–55° C. to avoid the possibility of any sucrose inversion, especially towards the end of distillation when the bulk was reduced to a few cc. of a syrupy fluid. This latter was then taken up in water by washing it several times, with a little warm water and clean sand each time. The washings thus collected were finally clarified.

Among the many clarifying agents used by earlier investigators (1), basic lead acetate was most extensively applied, followed by one of a number of such reagents as carbonates, sulphates, oxalates of sodium or potassium to remove the excess lead. Loss of reducing sugars, especially of fructose present in the extract, was noticed under such treatment by many workers, and was attributed to a destructive action of the lead salt on fructose (7). ENGLIS and TSANG (2) had experimentally shown that this loss in reducing sugars from tissue extracts, clarified with basic lead acetate, was due to the nature of the deleading agents used, and not to the lead salt. In view of the facts hitherto mentioned, many trials on clarifying carrot tissue extract were carried out, using some of the reagents most commonly used for this purpose. Two precipitants, namely basic and neutral lead acetate, were chosen. Each one of the two reagents was tried on two aliquot samples of the same extract.

TABLE I

REDUCING POWER OF THE CLEAR PLANT EXTRACT EXPRESSED AS GRAMS "GLUCOSE" PER 100 GRAMS FRESH WEIGHT OF CARROT TISSUE

CLARIFYING REAGENTS EMPLOYED	TOTAL REDUCING VALUE OF THE EXTRACT	R.V. OF THE EXTRACT AFTER FERMENTATION	R.V. OF THE FERMENTABLE PORTION OF THE EXTRACT
THE FERRICYANIDE METHOD			
(1) Basic lead acetate + disodium hydrogen phosphate	3.468	0.387	3.081
(2) Basic lead acetate + potassium oxalate	3.287	0.387	2.900
(3) Neutral lead acetate + disodium hydrogen phosphate	3.761	0.376	3.385
(4) Neutral lead acetate + potassium oxalate	3.525	0.382	3.143
THE COPPER METHOD			
(1) Basic lead acetate + disodium hydrogen phosphate	3.294	0.340	2.954
(2) Basic lead acetate + potassium oxalate	3.220	0.336	2.884
(3) Neutral lead acetate + disodium hydrogen phosphate	3.519	0.323	3.196
(4) Neutral lead acetate + potassium oxalate	3.229	0.312	2.917

The excess of lead was removed from each member of the two pairs of aliquots with one or the other of the two deleading agents chosen; namely, disodium hydrogen phosphate and potassium oxalate. The reducing power of the aliquots was then estimated before and after fermenting the clarified extract with yeast. The estimation was carried out with both the ferricyanide (3) and the copper (12) methods, elaborated and modified in this laboratory for measuring the reducing power of plant extracts.

The results presented in table I show that the application of lead acetate, either basic or neutral, followed by disodium hydrogen phosphate consistently resulted in higher reducing values than when potassium oxalate was used in place of phosphate; especially for the fermentable fraction of the extract which includes all the reducing sugars. These results are in accord with the conclusion of ENGLIS and TSANG (2).

Accordingly the treatment with basic lead acetate as a precipitant, followed by disodium hydrogen phosphate as a deleading agent, was adopted for the work on carrot tissue extracts.

ESTIMATION OF INDIVIDUAL CARBOHYDRATES IN CARROT TISSUE EXTRACT AND THE METHODS EMPLOYED

Two methods, previously modified and elaborated in this laboratory, for the estimation of reducing substances in plant extract, were used.

THE FERRICYANIDE METHOD.—This is a modification of HAGEDORN and JENSEN's (3) method which was first introduced and modified in this laboratory by Hanes, and finally standardized by HULME and NARAIN (6) for the estimation of pure reducing sugars in solution.

THE COPPER METHOD.—This is a modification of SHAFFER and HARTMAN's (12) method which was later standardized by MASKELL and NARAIN (work hitherto unpublished). The principal modification consists in the use of a less alkaline solution and the removal of oxygen from the mixture of extract and copper reagent before boiling, by means of a stream of nitrogen gas. Under these conditions there is no re-oxidation of reduced copper during boiling, and the thiosulphate titration value is proportional to the amount of sugar present, up to a maximum of 1.5 mg. of the reducing substance (hexose) to be estimated.

Each of the two methods proved to have some characteristic merits. The ferricyanide has an advantage over the copper method in the fact that it is not affected by any oxygen that may be present in the experimental solution. Also larger amounts of reducing substances can be estimated by the ferricyanide than by the copper method; the upper limits being 3.0 and 1.5 mg., respectively. On the other hand, the copper method, relative to monosaccharides, is less sensitive to reducing disaccharides than is the ferricyanide method. Consequently, with the copper method, the rise in the reducing value after the hydrolysis of disaccharides, will be relatively greater and hence more accurately estimated, than with the ferricyanide method.

In view of the differences between the two methods and the advantages

each one has over the other in some respects, both were simultaneously used during the early part of this work. When the identity of the individual members of the carbohydrate group present in carrot tissue became well established, the copper method was chosen, being more suited to carry on with the rest of the estimations for this work (9). However, the ferricyanide method was called on whenever the necessity arose, to check results obtained by the copper method.

ESTIMATION OF INDIVIDUAL CARBOHYDRATES IN CARROT TISSUE EXTRACT

THE ALDOSES AND KETOSES OR APPARENT HEXOSES.—Among the methods used for the estimation of aldose sugars is the iodometric one, elaborated by ROMIJN (10) and based on the oxidation of aldoses by iodine in alkaline solution. MASKELL and NARAIN (unpublished data) found this method to give correct results for mixtures of pure sugars; for leaf extracts, however, the figures for aldose sugars, indicated by the iodine used up, were invariably much too high.

In the present work, the method was tested on carrot tissue extract. The residual iodine in the experimental samples was titrated with $N/10$ sodium sulphite and the difference between the values thus obtained and a blank estimation, gave values in terms of Na_2SO_3 equivalent to the I_2 reduced by the experimental solutions. Table II contains a few sets of results which represent values for the aldoses estimated by the iodometric, ferricyanide, and the copper methods. In using the two latter methods for the estimation of the aldoses, samples of the extract were treated with iodine as mentioned above, merely to remove the aldoses; then the ketoses, presumably left untouched, were estimated. The difference between the reducing value of the extract both before and after the iodine treatment gave the value for the aldoses. The extract used for this investigation was previously hydrolyzed with N 2 HCl for increasing lengths of time at 60° C. during the course of elaborating techniques for sucrose estimation. The figures obtained and presented in table II are in accord with NARAIN's (8) results working on pure sugars and ivy leaf extracts.

In view of the excessively high estimates obtained for apparent glucose (aldoses) with the iodometric method, the use of this method for accurate estimation of these sugars was ruled out. It not only falsifies the figures for aldoses, but it also leads to fictitious results for ketoses which are to be obtained by subtracting the aldose value from the total reducing value of the two, estimated jointly by either the ferricyanide or the copper method. Despite the limitations of the iodometric method and in view of the fact that ketoses are hardly oxidized by iodine under these conditions, the iodometric method was made use of merely as a means of getting the aldoses present in the extract inactive by oxidation, leaving the residual ketoses to be estimated by either of the standard methods. The coupling of the iodometric method with the standard reduction methods, proved very valuable in obtaining

reliable and accurate results. Trials for recovering pure sugars (glucose, fructose, and sucrose) added to the tissue extract proved satisfactory.

THE TRUE HEXOSES—GLUCOSE AND FRUCTOSE.—Some reducing substances that may be mistaken for glucose and fructose proved to be always present in carrot tissue extract; since these substances proved to be completely resistant to yeast fermentation, the yeast fermentation technique was applied, as described previously, to fractionate the reducing substances in the extract into non-fermentable and fermentable fractions. This latter was taken to represent the joint value of true glucose and true fructose. Practically in every case where the yeast fermentation technique was applied, and before applying the estimates of the non-fermentable substance to the interpretation of experimental results, a test was carried out to ensure the complete removal of the fermentable sugars, without prolonging the standard fermentation

TABLE II

THE EXPERIMENTAL MATERIAL WAS HYDROLYZED WITH $N/2$ HCl AT $60°$ C. FOR VARIED LENGTHS OF TIME BEFORE OXIDATION WITH IODINE

MATERIAL USED	GRAMS APPARENT GLUCOSE PER 100 GRAMS F. WT. ESTIMATED BY THE:		
	IODOMETRIC	FERRICYANIDE	COPPER
20-cc. extract hydrolyzed for 4 minutes	3.735	2.617	2.720
8 "		2.123	2.991
16 "	2.820	2.655	2.220
30 "	3.100	2.800	2.632
60 "	4.230	2.515	2.861
Results of another set from a duplicate extract			
20-cc. extract hydrolyzed for 4 minutes	5.072	2.158	3.365
8 "	4.795	2.656	3.487
16 "	5.640	3.578	3.139
30 "	5.215	3.559	3.347
60 "	4.650	3.325	3.295

period. This involved the fermentation of an aliquot sample of the extract to which a known amount of pure glucose or pure fructose was added. In almost all cases, the results obtained after fermenting the entire and mixed extracts, agreed fairly well. In cases when such an agreement was not attained, however, the quantity of yeast employed was increased to effect such agreement.

Although the joint value for true glucose and true fructose present in carrot tissue extract could be obtained according to the technique so far described, no accurate separate values for the two hexoses could possibly have been obtained without firstly ascertaining whether this non-fermentable fraction belonged to the aldo or to the keto type of reducing substances, or whether it was a mixture of the two; and secondly, estimating the value of each component of a mixture.

ALDO AND KETO NON-FERMENTABLE SUBSTANCES.—To separate the non-fermentable substances into aldo and keto fractions, 20- to 30-cc. samples of the

extract were fermented under the conditions of the standard method, and the reducing value of the fermented extract was estimated. Samples of the fermented extract were then oxidized with iodine and the reducing value of the oxidized solution was again measured. This latter value was taken to represent the .keto non-fermentable substance, and the difference between this and the total value (before oxidation but after fermentation) to represent the value for the aldo non-fermentable substance. By subtracting these two figures from those representing the total ketoses and total aldoses (apparent hexoses, respectively) the values for true fructose and true glucose were obtained. This procedure with a slight modification, was duplicated for confirmation. The same steps were performed, only with their sequence reversed; namely, oxidation preceding fermentation. With both sequences fairly good agreements were obtained. The results obtained at this stage and throughout the present work, indicated that the non-fermentable matter present in carrot root extract is composed of two different types; i.e., a keto and an aldo, and that the keto type constituted the major part; at times the aldo type was entirely absent. Nothing like a constant ratio between the relative values of these two substances did exist at any time. This realization, coupled with the fact that the value of the non-fermentable fraction in the extract was by no means too low to be ignored, rising at times to more than 10% of the value for true hexoses, necessitated going through the tedious processes of their fractionation whenever the estimation of true sugars was required.

Before passing to the estimation of other members of the carbohydrate group present in carrot tissue extract, a few trials were carried out to check the reliability of the figures so obtained in representing the true hexoses in that extract. Recovery of pure fructose added to the extract was tried, both before and after treating the mixture with iodine. Within the limits of experimental error, 100% recovery was obtained, suggesting that nothing in the carrot tissue extract, nor the treatment with iodine, affects the recovery of pure sugar added to the extract in any measurable degree, and hence the genuineness of the figures for glucose and fructose present in this extract may be accepted.

THE DISACCHARIDES—SUCROSE.—The method adopted in this laboratory and applied to pure sucrose and leaf extracts was based on hydrolyzing the material to be analyzed for sucrose, with half normal hydrochloric acid at 60° C., and measuring the reducing value after hydrolysis.

Since the value for sucrose in carrot tissue extract was to be obtained from the increase in the reducing value of the extract after hydrolysis with N/2 HCl at 60° C., the accuracy of such a value would necessarily depend on, firstly, whether the non-fermentable reducing substances in the extract would undergo any substantial change under the conditions of hydrolysis; and, secondly, whether any disaccharide, other than sucrose, may be present in the extract to augment the reducing value of that extract on hydrolysis.

A few trials were carried out to test the behavior of the non-fermentable

TABLE III

MATERIAL	REDUCING VALUES AS MGS. GLUCOSE IN 20 CC. TISSUE EXTRACT ESTIMATED BY:			
	THE FERRI-CYNIDE METHOD		THE COPPER METHOD	
	mg.		mg.	
20-cc. extract fermented	15.80		13.6	
20-cc. extract hydrolyzed and fermented	10.00	37*	9.4	31*

* Percentage loss.

reducing substances under these conditions. Samples of the extract were fermented and other aliquot samples were hydrolyzed and then fermented. Results obtained and recorded in table III clearly show a consistent loss of the non-fermentable material; a loss which suggests that the material in question had either undergone partial destruction under the prevailing conditions, or had been partially hydrolyzed into some substance fermentable by the yeast used. To clear up this point, samples of the extract were fermented, and a part of the fermented extract hydrolyzed with N/2 HCl at 60° C. The result thus obtained and recorded in table IV indicates that the non-fermentable reducing matter in carrot tissue extract undergoes partial destruction under the conditions of hydrolysis with N/2 HCl. Although the loss in the non-fermentable substance amounted to over 30% at times, the total value of this substance relative to the total reducing power of the carrot tissue extract (after hydrolysis) is quite small, the allowance for this destruction can be safely ignored without appreciably affecting the results for sucrose.

To find out whether sucrose was present and if so, whether it was the only disaccharide that occurs in carrot tissue, a study of the hydrolysis time curve was carried out. Such a curve would also indicate the period necessary for hydrolysis to attain completion. For this purpose two comparable tissue samples A and B were separately extracted and clarified. From the clear

TABLE IV

MATERIAL	REDUCING VALUES AS MGS. GLUCOSE IN 20 CC. TISSUE EXTRACT ESTIMATED BY:			
	THE FERRI-CYANIDE METHOD		THE COPPER METHOD	
	mg.		mg.	
20-cc. extract fermented	15.2		12.6	
20-cc. extract hydrolyzed and fermented	12.2	20*	10.0	20*

* Percentage loss.

extracts, two sets of samples, one set for each extract, were hydrolyzed with N/2 HCl at 60° C. for varied lengths of time, and their reducing values estimated both before and after hydrolysis with both the two standard methods. The results obtained are presented in table V. It is evident from these results, that the total reducing value of the extract rose rapidly with time, until a level was reached after 16 minutes, beyond which time no further increase or decrease took place.

TABLE V

ANALYSIS OF TWO DUPLICATE EXTRACTS A AND B
RESULTS ARE EXPRESSED AS GRAMS APPARENT HEXOSES PER 100 GRAMS FRESH WEIGHT

METHOD OF ESTIMATION	MATERIAL USED		TOTAL R.V. AS APPARENT GLUCOSE (1)	APPARENT FRUCTOSE (2)	APPARENT GLUCOSE (3)
			gm.	*gm.*	*gm.*
The copper	Unhydrolyzed extract	A. B.	3.780 } 3.783 3.785	1.710 } 1.775 1.840	2.070 } 2.008 1.945
	Hydrolyzed at 60° C. for:				
	4 minutes	A. B.	5.375 } 5.698 6.020	2.788 } 2.759 2.725	2.587 } 2.941 3.295
	8 "	A. B.	6.160 } 6.060 5.960	3.022 } 3.116 3.210	3.138 } 2.944 2.750
	16 "	A. B.	6.355 } 6.338 6.320	3.140 } 3.074 3.007	3.215 } 3.264 3.313
	30 "	A. B.	6.250 } 6.338 6.425	3.125 } 3.160 3.195	3.125 } 3.178 3.230
	60 "	A. B.	6.310 } 6.365 6.420	2.808 } 2.979 3.150	3.502 } 3.386 3.270
The ferricyanide	Unhydrolyzed extract	A. B.	3.995 } 4.164 4.332	2.180 } 2.262 2.343	1.815 } 1.902 1.989
	Hydrolyzed at 60° C. for:				
	4 minutes	A. B.	5.880 } 6.420 6.960	3.338 } 3.194 3.050	2.542 } 3.226 3.910
	8 "	A. B.	6.930 } 6.981 7.032	3.562 } 3.556 3.550	3.368 } 3.425 3.482
	16 "	A. B.	7.465 } 7.415 7.365	3.575 } 3.505 3.435	3.890 } 3.910 3.930
	30 "	A. B.	7.135 } 7.288 7.440	3.587 } 3.599 3.607	3.548 } 3.691 3.833
	60 "	A. B.	7.185 } 7.378 7.570	3.475 } 3.388 3.300	3.710 } 3.990 4.270

To identify and fractionate the hydrolysis products, the reducing values of the extract, before and after hydrolysis, were resolved into ketose and aldose fractions, and the results obtained were again recorded in table V under columns 2 and 3, respectively.

Since the maximum reducing value of the hydrolysis products was attained after 16 minutes and remained constant beyond that time, the values obtained for each set (A and B) in 16, 30, and 60 minutes were treated as comparable estimates of the hydrolysis products, and accordingly their mean

for each set, as well as for the two sets combined, was taken to give a more accurate estimate for 100% hydrolysis. In table VI, column 2, the means of the three last estimates for the hydrolysis products, differentiated into aldoses and ketoses are presented. In the same table, column 1, are also tabulated the aldoses and ketoses initially present in the extract. Since no allowance for destruction in the non-fermentable matter was involved at this stage, the difference between the corresponding values in columns 1 and 2 was taken to represent true glucose and true fructose that resulted from the hydrolysis of the substance or substances under investigation. These values for true glucose and fructose are shown in column 3.

The results obtained by the copper method (table VI) suggest that the products of hydrolysis, within the limits of experimental error, are composed of equal quantities of true glucose and true fructose. By the ferricyanide

TABLE VI

REDUCING VALUES AS GRAMS HEXOSE PER 100 GRAMS FRESH WEIGHT

METHOD OF ESTIMATION	MATERIAL	APPARENT HEXOSE				INCREASE IN TRUE HEXOSES DUE TO HYDROLYSIS	
		INITIAL		AFTER HYDROLYSIS			
		(1)		(2)		(3)	
		KETOSES	ALDOSES	KETOSES	ALDOSES	FRUCTOSE	GLUCOSE
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Copper	Set A	1.71	2.07	3.02	3.28	1.31	1.21
	Set B	1.84	1.95	3.12	3.27	1.28	1.32
	Mean (A & B)	1.775	2.01	3.07	3.275	1.295	1.265
Ferricyanide	Set A	2.18	1.82	3.55	3.72	1.37	1.90
	Set B	2.34	1.99	3.45	4.01	1.11	2.02
	Mean (A & B)	2.26	1.905	3.50	3.865	1.24	1.96

method, however, glucose is consistently higher than fructose; a discrepancy which is quite inexplicable and must be ascribed to some failing in that method rather than to a substance in the extract responsible for this excess of glucose. This assumption is substantiated by the fact, as mentioned earlier, that the copper method relative to hexose is less sensitive to disaccharides than is the ferricyanide method; hence it follows that in case of the presence of a substance of this category in the extract, the copper method should give—after hydrolysis—higher reducing values relative to the initial reducing power of the extract, than would the ferricyanide method. In no instance was this the case; on the contrary, the ferricyanide method invariably gave higher results. Nevertheless, an attempt was made to find a cause for this disparity between the two components (glucose and fructose) of the hydrolysis products, as estimated by the ferricyanide method; a cause which was thought to lie probably in the oxidation reagents and their possible interference with the estimation of ketoses—and consequently the aldoses. After many fruitless efforts, through which it was well established that the oxida-

tion reagents did in no way interfere with the estimation of the reducing power of carrot extracts no such cause was discovered to account for the behavior of the ferricyanide method in this respect.

Since the rate of hydrolysis of any material under any given set of conditions is a fairly good index to its identification, the rate of hydrolysis of carrot root extract under these conditions was calculated, in the hope of shedding some light on the nature of the substance in question. The results of calculation are shown in table VII. The mean value of the last three esti-

TABLE VII

PERCENTAGE AND RATE OF HYDROLYSIS

TIME OF HYDROL-YSIS	METHOD OF ESTIMATION					
	THE COPPER METHOD			THE FERRICYANIDE		
	INCREASE IN R.V. GLU. + FR.	HYDROLYSIS		INRCEASE IN R.V. GLU. + FR.	HYDROLYSIS	
		PERCENT-AGE	RATE*		PERCENT-AGE	RATE
		%			%	
4 minutes	1.915	74.7	0.37	2.257	70.6	0.31
8 "	2.278	88.9	"per	2.818	88.2	"per
16 "	2.555	99.7	minute"	3.252	101.7	minute"
30 "	2.555	99.7		3.124	97.8	
60 "	2.583	100.7		3.214	100.6	
Mean of 16, 30, and C0 min-utes	2.564	100.0		3.197	100.0	

* Rate of hydrolysis was worked out according to the equation (4)

$$K = \frac{1}{t} \log \frac{a}{a-x}$$

Where K is rate of hydrolysis in t time: a the amount of cane sugar present at the start, and x the amount of cane-sugar that is hydrolyzed.

mates for the hydrolysis products (after 16, 30, and 60 minutes) was taken to represent 100%. Accordingly, percentage hydrolysis after varied lengths of time, and also the rate of hydrolysis [after HARVEY (4)] were calculated.

It is evident that the results obtained for the products of hydrolysis at 60° C. with N/2 HCl, show that:

1. The hydrolysis time curve attained a maximum value after 16 minutes, beyond which time it kept practically level.

2. The hydrolysis products are composed entirely of glucose and fructose, as measured by the copper method.

3. The rate of hydrolysis of the extract is of the same order as that for pure sucrose under the same conditions.

Such conclusions strongly suggest that sucrose is the only disaccharide present in carrot tissue extract. Accordingly, the technique adopted for the estimation of sucrose in this work, was based on treating the extract with N/2 HCl at 60° C. for 30 minutes.

MALTOSE.—The method which has been used in this laboratory by MAS-
KELL and NARAIN (unpublished data) for the estimation of maltose, based
on the hydrolysis of this sugar with N HCl at 100° C. and allowing for the
destruction of the hydrolysis products at the rate of 1% per hour, was tried
on carrot tissue extract; also hydrolyzing the extract with 0.5% Taka-
diastase was attempted. Results obtained are recorded in table VIII, to-
gether with the reducing value of the same extract after N/2 HCl hydrolysis
at 60° C. The increase due to hydrolysis after both the treatment with N/2
HCl at 60° C. and with 0.5% Taka-diastase is practically the same. The low
results obtained after hydrolysis with N HCl at 100° C. might be due to a
greater rate of destruction than has been allowed for. These results further
confirm the conclusion, already arrived at; namely, that sucrose is the only
disaccharide present in carrot root tissue.

TABLE VIII

REDUCING VALUES OF CARROT TISSUE EXTRACT AS GRAMS TRUE HEXOSES PER 100 GRAMS
FRESH WEIGHT BEFORE AND AFTER DIFFERENT TREATMENTS*

REDUCING VALUES	HYDROLYZING AGENT:		
	N/2 HCl AT 60° C.	N HCl AT 100° C.	0.5% TAKA-DIASTASE AT 35° C.
	gm.	gm.	gm.
T.R.V. before hydrolysis	4.165	4.155	4.200
T.R.V. after "	7.365	6.906	7.403
Increase " "	3.200	2.751	3.203

* Figures shown in this table are means of values obtained for the duplicate extracts
A and B by the ferricyanide method.

To conclude: here follows a short account of the steps followed for a com-
plete analysis sheet of individual carbohydrates, present in the 80% alcohol
extract of carrot tissue, according to the technique hitherto discussed:

STEPS	TREATMENT	RESULTS
A.	Extract directly estimated	Direct total reducing value
B.	Extract fermented with yeast and estimated	Total non-fermentable reducing matter
C.	Fermented extract oxidized with iodine	Keto non-fermentable reducing matter
D.	Extract oxidized with iodine	Total direct reducing ketoses
E.	Extract hydrolyzed with N/2 HCl at 60° C.	Total reducing value after hydrolysis
F.	Hydrolyzed extract oxidized with iodine	Total reducing ketoses after hydrolysis

Thus

A–B = Total fermentable reducing matter, or true hexoses
B–C = Aldo non-fermentable reducing matter
A–D = Total direct reducing aldoses

A–D–(B–C) = Fermentable direct reducing aldoses or true glucose
D–C   = Fermentable direct reducing ketoses or true fructose
E–A   = Total increase in fermentable reducing matter or the in-
             crease in glucose and fructose due to inversion
E–F   = Total reducing aldoses after inversion
F–D   = Increase in fructose due to inversion
E–F–(A–D) = Increase in glucose due to inversion

All the results obtained according to the above scheme represent reducing values in terms of glucose, and accordingly (D–C) and (F–D) in order to represent fructose, must be corrected by adding 3%; fructose being that amount lower in its reducing power than glucose, as estimated by the copper method.   Hence it follows:

$$\frac{(D-C) \times 100}{97} = \text{Initial fructose}$$

$$\frac{(F-D) \times 100}{97} = \text{Increase in fructose after inversion}$$

$$\frac{(F-D) \times 100}{97} + E-F-(A-D) = \text{Sucrose as invert sugar}$$

$$\frac{342}{360} \times \left(\frac{(F-D) \times 100}{97} + E-F-(A-D) \right) = \text{Sucrose as such}$$

Summary

1. Carrot tissue was extracted with 80% boiling alcohol.   The aqueous extract was cleared with basic lead acetate and disodium phosphate.

2. Using the two reduction methods (the copper and ferricyanide) glucose, fructose, and sucrose were found and estimated in the clear extract. Maltose was entirely absent.

3. Trials for fermenting various sugars with ordinary baker's yeast were attempted.   Glucose, fructose, and sucrose were completely fermented in less than three hours.   Maltose was more resistant than the three mentioned sugars.   It was almost equally rapidly fermented when a solution of Taka-diastase was added to the yeast suspension.   The pentoses, arabinose and xylose, were not attacked by the yeast used.

4. The yeast technique was applied to carrot tissue extract for separating the non-fermentable reducing matter which was generally taken for reducing sugars.

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RESPIRATION AND NITROGEN METABOLISM OF WHOLE AND SLICED RADISH ROOTS WITH REFERENCE TO THE EFFECT OF ALTERNATION OF AIR AND NITROGEN ATMOSPHERES[1]

HUSEIN SAÏD AND E. D. H. EL SHISHINY

(WITH FOUR FIGURES)

Introduction

It has long been known that wounded plants respire more intensely than intact ones (3, 5, 6, 13, 14, 17, 18, 22, 23, 29, 30, 31, 32).

AUDUS (1, 2), and GODWIN (10) reported that handling of plant parts, without apparent wounding, causes great increase in their rate of respiration. The stimulus that causes this rise in respiration arises apparently from deformation of cells by bending or rubbing. MILLER (19) states that the marked increase in the output of carbon dioxide following injury is probably due to the escape of the gas that has accumulated in the inter-cellular spaces of the tissues. There is no doubt that injury of bulky organs renders the atmospheric oxygen more accessible across the tissues (3, 18, 19, 27, 32).

TURNER (31) observed that when carrot roots are sliced into 1-mm.-thick discs, the latter usually give a high initial rate of respiration which exhibits wide variations but with general tendency to fall. After 50 hours, the fall in respiration becomes more regular until after 150–200 hours, a rate is reached which is about the same as that of intact carrot root. The high initial respiration of the carrot slices is ascribed to stimulation caused by cutting, washing, and handling.

BENNET-CLARK and BEXON (3) found that with continued washing in aerated tap water, the respiration rate of beet root slices rises from a very low value shortly after cutting to a high maximum value after some 300 hours' washing, a value which is then more or less maintained for another 350 hours. The authors suggest that the rise in the respiration of the slices and the maintenance of high rate for many days is due to less restricted gaseous exchange and to mechanical injury and wound stimuli which may induce metabolic changes leading to an increased concentration of respirable substrate or of respiration enzymes.

STILES & DENT (30), measuring the respiration activity of discs from various storage tissues, found that on first cutting out the slices the respiration starts low and rises slowly in aerated running tap water to a much higher level which may be maintained constant for a time, but from which it slowly diminishes. These two authors agree with BENNET-CLARK and

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[1] This is the fourth of a series of papers on the general subject of Researches on Plant Metabolism.

BEXON (3) in the explanation of the gradual rise of the respiration activity as a consequence of separation of thin slices from a storage organ, and they further consider the subsequent fall in respiration rate to be due to exhaustion of the respirable substrate or to the slow development of some depressant factor.

Some of the metabolic changes that occur as a result of injury of plant organs have been studied. Thus GRUSS (12) observed that wounding of potato induced sugar accumulation and increased oxidizing enzymes and diastatic activity in the cells around the wound. FRIEDRICH (9) noted in the cells bordering the cut surfaces of potato an increase in the total nitrogen and reducing sugars. HOPKINS (15) showed that wounding caused disappearance of starch and increase in sugar content; the latter resulted in increase of respiration activity. LUTMAN (18), on the other hand, was of the opinion that the increase in sugar concentration had no relation with the increase in respiration of wounded tissues.

Recent work seems to indicate that respiration may be more closely connected with nitrogen metabolism than with carbohydrate changes (11, 16, 21, 28). ZALESKI (34) claimed that wounded storage organs always increased their protein content, presumably at the expense of their soluble nitrogen.

The aim of the present paper is to investigate the changes in the respiration activity resulting from the separation of thin slices from roots of *Raphanus sativus Aegyptiacus,* and to see if these changes would be accompanied by variations in the nitrogen fractions of the plant.

Experimentation and results

The carbon dioxide given off in respiration was absorbed in Pettenkofer tubes and then estimated titrimetrically. By the use of a battery of Pettenkofer tubes and a commutator designed by F. F. Blackman, a continuous day and night record of respiration could be obtained. The methods used for the estimation of the various nitrogen fractions have already been referred to in a previous paper (25).

Mature radish plants were usually brought to the laboratory early in the morning; the leaves were removed, the roots thoroughly washed with tap water and then wiped dry. In experiments on whole roots, the fresh weights of the roots were first determined; and then the cut ends were sealed with wax of low melting-point. Slicing of the roots was always carried out by means of a special sledge microtome. The following experiments are typical representatives of many repetitions and the results reported have been fully confirmed.

FIRST EXPERIMENT

RESPIRATION AND NITROGEN METABOLISM OF WHOLE RADISH ROOTS.—Four more or less similar roots were sterilized by means of freshly prepared solution of 6.66 per cent of calcium hypochlorite as recommended by WILSON

(33) and PEARSALL and BILLIMORIA (20), and then washed with sterilized distilled water. After that two roots were extracted with boiling distilled water and immediately analyzed for the various initial nitrogen fractions. The other two roots were placed in two respiration chambers which were then submerged to the neck in a water-bath whose temperature was kept constant at 25° C. by means of a toluene-mercury thermo-regulator. A carbon-dioxide-free air was passed through the chambers at a rate of two liters per chamber per hour. Allowance of two hours was always given for the sweeping of all the carbon dioxide present in the apparatus, and also for the plant material to adapt itself to the temperature of the bath. After that the carbon-dioxide outputs by the two roots were estimated at 8-hour intervals for 120 hours. At the end of the experimental period, the two samples were extracted in boiling water and analyzed for their nitrogen fractions.
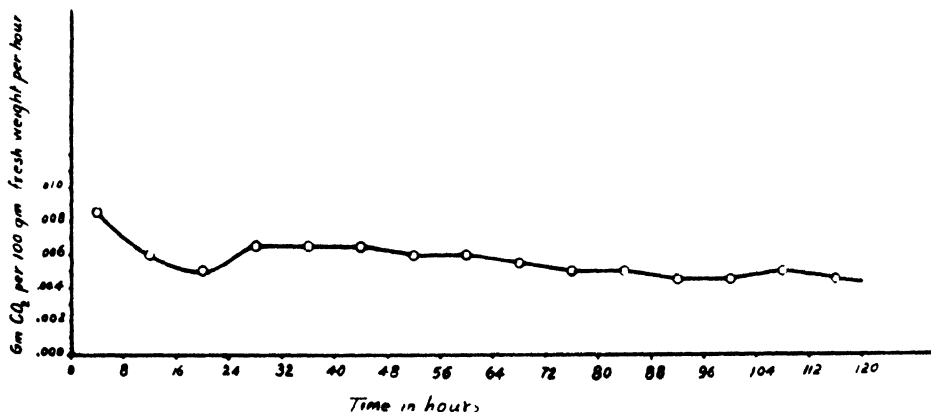
FIG. 1. Drift in the respiration rate of mature whole radish roots.

RESPIRATION OF WHOLE ROOTS.—The average carbon-dioxide output of the two samples is represented graphically in figure 1, being calculated as grams CO_2 given off by 100 grams fresh weight of roots per hour. The results of this as well as many similar experiments showed that the respiration of whole radish roots always proceeds at a low but steady rate for prolonged periods.

ANALYSIS OF TISSUES.—The results obtained from the analysis of the radish roots for their nitrogen fractions are recorded in table I. It is clear from this table that very little change, if any, takes place in the nitrogen fractions during 120 hours' starvation of mature radish roots. It is interesting to note that small but consistent inverse changes are always observed in the ammonia and amide-N fractions.

SECOND EXPERIMENT

RESPIRATION OF WHOLE AND SLICED ROOTS.—The respiration of two whole roots was determined in the usual way as in the first experiment except that the carbon-dioxide outputs were estimated at 5-hour intervals. After 45 hours, when the steady drifts in the rates of respiration of the two samples

TABLE I

CHANGES IN THE NITROGEN FRACTIONS OF WHOLE RADISH ROOTS RESPIRING IN AIR
FOR 120 HOURS, ALL BEING CALCULATED IN MILLIGRAMS PER
100 GRAMS FRESH WEIGHT

SAMPLES	TIME OF ANALYSIS IN HOURS	NITRATE N	AMMONIA N	AMIDE N	OTHER N	TOTAL SOLUBLE N	PROTEIN N	TOTAL N
	hrs.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
A	0	40.5	4.5	5.8	66.6	117.4	45.2	163.6
B	0	45.2	5.1	5.9	63.5	119.7	48.7	168.4
C	120	45.5	2.7	9.2	60.8	118.2	44.0	162.2
D	120	44.9	3.6	7.4	66.1	122.0	42.5	164.5

were ascertained, one root (sample A) was sliced, and the slices were sus-
pended in 200 ml. of sterilized distilled water through which air was passed.
The carbon-dioxide outputs by the whole and sliced roots were then followed
for another period of 80 hours. The drifts in the respiration rates of both
whole and sliced roots throughout the experimental period of 125 hours are
presented in figure 2, all being calculated as grams carbon dioxide given off
per 100 grams fresh weight of tissue per hour.

During the first 45 hours the two roots showed similar low but steady
respiration rates as in the previous experiment. The period immediately
following the slicing of sample (A) was characterized by irregular increases
in the carbon-dioxide output by the sliced root. The respiration of the other
whole root continued at a low steady rate with very slight tendency to fall.

It is interesting to note that the respiration rate of the sliced root reached
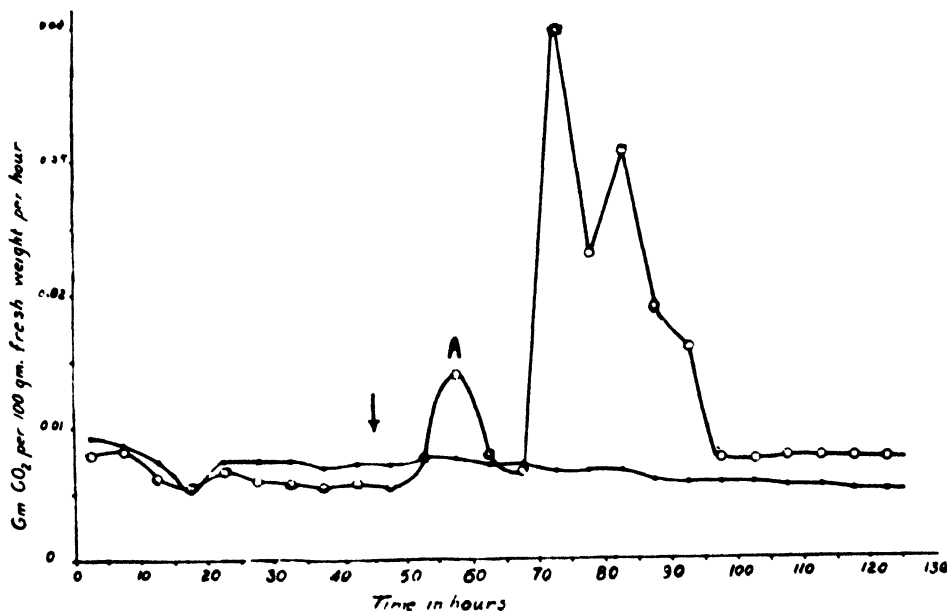
FIG. 2. Respiration rates of whole and sliced radish roots; arrow indicates the point
at which slicing of sample A took place.

its maximum 30 hours after slicing and then subsided fairly rapidly for 20 more hours, after which it reached a steady level course parallel to that of the intact root, though slightly higher. The results of this and many similar experiments agree well with the findings of STILES and DENT (30) using slices from various storage organs. It may be recalled here that BENNET-CLARK and BEXON (3) failed to record the decline in the respiration intensity of the slices after reaching its maximum, and TURNER (31) did not observe the rise of the respiration rate of slices from a low value to a maximum.

The rise in respiration rate obtained after cutting out the discs has been attributed to more free gaseous exchange (3, 19, 30, 32), to mechanical and wound stimuli (1, 2, 10, 18, 31) which may induce internal metabolic changes leading to increased concentration of respiratory substrate or of respiratory enzymes (3, 9, 12, 15, 30).

The fall in respiratory activity of the slices after reaching its maximum has been considered by STILES and DENT (30) as due to exhaustion of respiratory substrate or to slow development of some depressant factor. The former seems to be the more probable explanation since STILES and DENT (30) have shown that the respiratory quotient of beet root slices and other tissues decline from 1 down to 0.85 under conditions leading to starvation; and this has been attributed to the utilization of proteins after the depletion of the carbohydrate substrate. Relevant to this point it may be stated that feeding the radish root slices with sugar causes a great increase in the respiration intensity of these slices well above that of the slices in distilled water (25). It must be remembered that while the more free accessibility of oxygen across the root slices tends to increase their respiration, the exhaustion of the available respirable substrate opposes and may even mask the effect of oxygen. SAÏD and SHISHINY (25) have shown that the increase in respiration rate with increase in specific tissue surface is much greater in sugar-fed slices than in slices suspended in water.

THIRD EXPERIMENT

RESPIRATION AND NITROGEN METABOLISM OF SLICED RADISH ROOTS.—Previous work (24, 25) has shown that the slices most suitable for the study of respiration and metabolism are those of 1 mm. thickness. For the present experiment, therefore, four more or less comparable samples of radish root slices of 1 mm. thickness were prepared in the following way: two consecutive discs were dried between blotting papers and then halved; each half was placed at random in one of four tin boxes whose weights had been determined, this procedure continued until about 20 grams of slices were obtained in each box. The four samples were first washed with calcium hypochlorite solution and then with sterilized distilled water. Samples 1 and 2 were extracted in boiling water and analyzed for their initial nitrogen fractions immediately. Samples 3 and 4, on the other hand, were placed in two respiration chambers, each containing 200 ml. of sterilized distilled water through which carbon-dioxide-free air was passed, and their carbon-dioxide outputs

were measured at 5-hour intervals. After 50 hours, when the respiration reached its steady rate, sample 3 was removed, extracted, and then analyzed for the various nitrogenous fractions. Sample 4 was left to continue respiring for another period of 55 hours and then analyzed for its nitrogenous fractions.

RESPIRATION OF RADISH SLICES.—The respiration of sample 4 is graphically represented in figure 3, being calculated in grams carbon dioxide per 100 grams fresh weight of slices per hour. The results of this experiment confirmed those of the previous one in showing that on first separating the thin slices from the root, their respiration starts low, and then increases to a maximum rate which may be reached after 20 to 30 hours, depending upon the previous history of the plant organ and experimental conditions such as
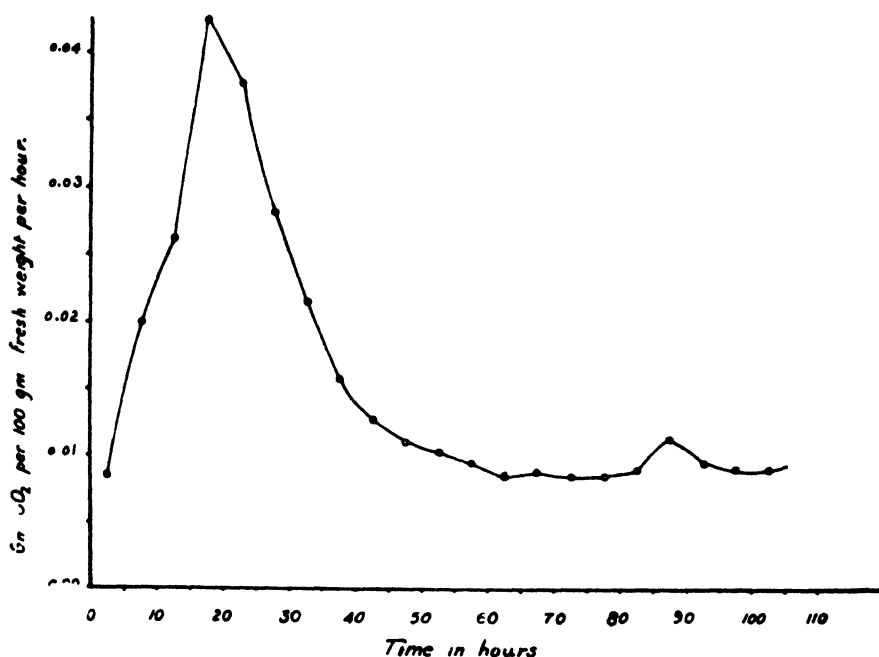
FIG. 3. Drift in the respiration rate of radish root slices.

temperature et cetera. This is followed by a subsequent rapid decline to a low steady rate which may be maintained for prolonged periods with very slight tendency to fall.

ANALYSIS OF TISSUES.—The results obtained from the analysis of the tissues for their nitrogenous fractions are given in table II. It is clear that the radish slices showed great changes in their nitrogenous fractions in the first 50 hours that followed cutting out the slices. After this period, very few changes took place in the nitrogenous fractions during the next 55 hours. It should be recalled that during this latter period the respiration of the slices was maintained at a low and steady level.

The essential change in the nitrogen metabolism that resulted from cutting out the discs was a decrease in the total soluble-N and an increase in the protein-N. The increase in the protein-N was too small to account for

the decrease in the total soluble nitrogen. Examining the mean value of the total nitrogen of the initial samples 1 and 2 and that of sample 3, it was found that sample 3 had lost 40.0 mg. nitrogen per 100 gm. fresh weight of tissues, which must have diffused out into the water medium. When the latter was analyzed, 39.3 mg. nitrogen could be recovered from the medium of 100 gm. fresh weight of slices.

It is interesting to note that although the radish slices lost a good deal of their nitrate nitrogen during the first 50 hours, yet no trace of nitrates, and insignificantly small amounts of ammonia-N, could be detected in the nitrogen recovered from the water medium. It seems, therefore, that the nitrates which disappeared from the tissues during the wounding metabolism must have been reduced and then transformed into some organic soluble

TABLE II

THE EFFECT OF ROOT SLICING ON NITROGEN DISTRIBUTION; ALL FRACTIONS ARE GIVEN IN MILLIGRAMS PER 100 GRAMS FRESH WEIGHT

Samples	Procedure	Nitrate N	Ammonia N	Amide N	Other N	Total Soluble N	Protein N	Total N	Nitrogen Recovered from the Water Media
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	Analyzed	39.5	3.7	9.7	66.4	119.3	59.6	178.9	
2	immediately	38.2	3.7	10.2	64.9	117.0	62.6	179.6	
3	50 hr. in distilled water	15.7	1.8	3.2	48.4	69.1	70.2	139.3	39.3
4	105 hr. in distilled water	14.8	1.2	6.8	44.8	67.6	70.6	138.2	40.5

form. The latter, together with the "other nitrogen" already present in the slices, might have been partly synthesized into protein and partly excreted into the external medium.

Another alternative that seemed plausible was that the protoplasm of the cut cells on the surfaces of the slices might have been disorganized and its proteins broken down into organic soluble compounds which appeared in the water medium. In the meantime, some nitrates, together with some of the "other nitrogen," were synthesized into proteins, thus compensating for all the protein of the disorganized cut cells and even exceeding the initial protein nitrogen content by 9 mg. per 100 gm. fresh weight of slices. Both alternatives might take place.

The amide nitrogen that disappeared from the slices during the first 50 hours of the experiment was most probably excreted into the medium. The increase in this nitrogen fraction during the later part of the experiment might have come from amidation of some amino-acids.

FOURTH EXPERIMENT

RESPIRATION AND NITROGEN METABOLISM OF RADISH SLICES IN ALTERNATING AIR AND NITROGEN ATMOSPHERES.—Eight more or less comparable samples, 20 grams each, of radish root slices of 1 mm. thickness were prepared. Two samples were extracted in boiling distilled water and then analyzed immediately for their initial nitrogen fractions. The remaining samples were washed in running tap-water for a few minutes and then placed in six respiration chambers, each containing 200 ml. of sterilized distilled water, and were all kept at a constant temperature of 25° C. Carbon-dioxide-free air was passed through the water in all the chambers at a steady rate of 2 liters per chamber per hour. The carbon dioxide given off by two representative samples was collected in two sets of Pettenkofer tubes and was
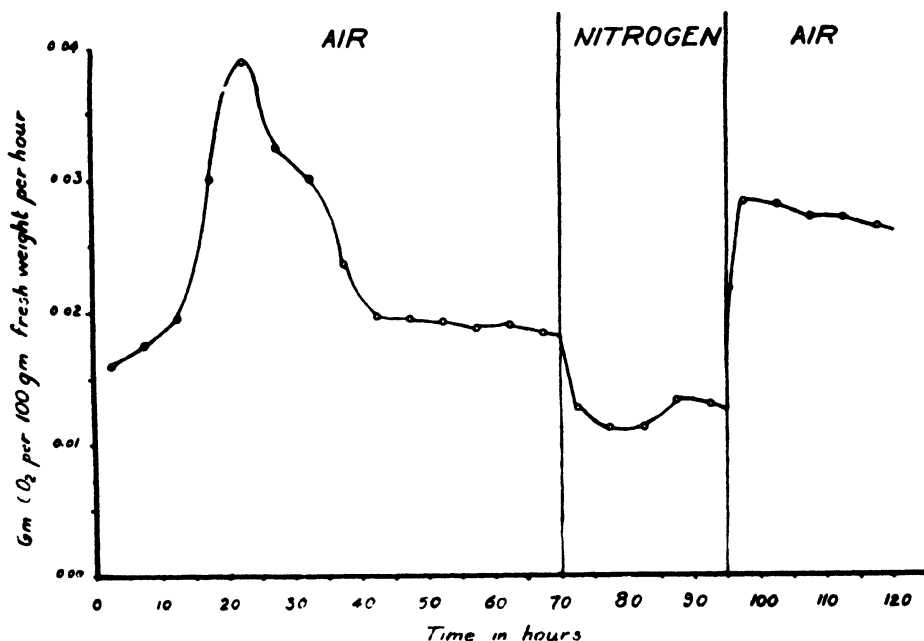
FIG. 4. Respiration rates of radish slices in alternating air and nitrogen atmospheres.

determined at 5-hour intervals. After 70 hours, two samples were drained, extracted and then analyzed for their nitrogen fractions. At the same time, pure nitrogen was passed through the remaining chambers, and the respiration rate was followed under the new conditions for a period of 25 hours. After that two more samples were extracted and analyzed while the last couple of samples were allowed to respire in carbon-dioxide-free air again for a further period of 25 hours, after which they were analyzed for their nitrogen fractions.

RESPIRATION OF THE SLICES.—The average drift in the respiration rate of the radish slices in alternating air and nitrogen atmospheres is illustrated in figure 4. It is clear that the slices first showed the respiration rate characteristic of wound-metabolism, which lasted about 45 hours, after

which the respiration rate declined to a low steady rate.   At the seventieth hour, the radish slices were transferred from air to nitrogen, and their respiration rate dropped rapidly to another level below that of the air-line respiration.   This lower carbon-dioxide output was more or less maintained during the period of anaerobiosis, and after 25 hours the slices were transferred back to air and their respiration rose very sharply above the air-line rate.   After that the respiration continued at a steady decreasing rate.   Other similar but prolonged experiments showed that the air-line respiration rate was usually reached about 48 hours after transference from nitrogen to air. It might be interesting to point out here that when the line of anaerobic respiration (fig. 4) was extrapolated back to the point of transference from air to nitrogen, the initial anaerobic respiration (INR) could be evaluated, and the ratio of INR to the aerobic respiration just before transference could thus be obtained.   This ratio was found to be 0.62, which clearly indicated that some oxidative anabolism occurred during aerobic respiration, but its value was much below that obtained by BLACKMAN for apple fruits (4). Similar results were reported by SHOUDHURY (26) and STILES & DENT (30).

ANALYSIS OF TISSUES

The analysis of the sliced samples for their various nitrogen fractions are recorded in table III.

THE BEHAVIOR OF TOTAL SOLUBLE AND PROTEIN NITROGEN

During the first period of 70 hours, the radish slices showed a decrease in their total soluble' nitrogen amounting to 38.3 mg. per 100 gm. fresh

TABLE III

THE EFFECT OF ALTERNATION OF AIR AND NITROGEN ATMOSPHERES ON THE NITROGEN
FRACTIONS OF RADISH SLICES, CALCULATED IN MILLIGRAMS
PER 100 GRAMS FRESH WEIGHT*

SAMPLES	PROCEDURE	NITRATE N	AMMONIA N	AMIDE N	OTHER N	TOTAL SOLUBLE N	PROTEIN N	TOTAL N	NITROGEN RECOVERED FROM THE WATER MEDIA
		mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1 2	Analyzed immediately	32.0	3.1	13.1	60.9	109.0	64.8	174.8	
3 4	70 hr. in air	17.1	1.7	5.5	46.4	70.7	78.6	149.3	30.0
5 6	70 hr. in air, then 25 hr. in nitrogen	17.2	2.4	3.8	50.0	73.4	75.9	149.3	30.3
7 8	70 hr. in air, 25 hr. in nitrogen, and then 25 hr. in air	13.7	2.4	2.5	60.1	78.7	72.9	151.6	29.6

* The values included in this table are means for duplicate samples.

weight of tissues. This decrease could be accounted for partly by some increase in the protein nitrogen and partly by the soluble organic nitrogen recovered from the external water media, thus adding further support to the results of the previous experiment.

During the following period of 25 hours, the plant tissues were respiring anaerobically, and there was a slight tendency of protein hydrolysis with corresponding increase in the soluble nitrogen content. Such protein hydrolysis, however, did not seem to be a direct result of anaerobiosis since it continued even when the nitrogen atmosphere was substituted by air in the last 25 hours of the experiment. This protein breakdown was most probably due to low carbohydrate content of the plant tissues.

The behavior of nitrate nitrogen

Similar to the findings of the previous experiment, the greatest change in the nitrate nitrogen fraction took place during the early period that followed the slicing of the roots. All the nitrates that disappeared from the tissues were not detected in the medium and were presumably synthesized into some organic form. During the 25 hours of anaerobiosis, there was no apparent change in the nitrate content of the tissue slices. It should be recalled that this period was characterized by the lowest respiration rate; and assuming that nitrate reduction involved the utilization of energy of respiration, then under anaerobic conditions the respiration must have been too low to produce the energy necessary for such nitrate reduction. Alternatively, the absence of oxygen from the atmosphere might have inhibited the action of the nitrate-reducing enzyme system [cf. ECKERSON (7, 8)].

Transference of the radish slices from nitrogen back to air for another period of 25 hours resulted in a disappearance of 3.5 mg. of nitrate nitrogen per 100 gm. fresh weight of tissues. The high respiration obtained on transference from nitrogen to air might have afforded enough energy for the resumption of nitrate reduction, or alternatively, the reducase enzyme system might have been reactivated by the oxygen of the atmosphere.

The behavior of "other nitrogen"

During the first period of 70 hours in air, this nitrogen fraction lost 14.5 mg. per 100 gm. fresh weight of slices which probably together with the nitrogen obtained from nitrate reduction, contributed partly to the protein synthesis and partly to the nitrogen excreted into the water medium. The next period of 25 hours in nitrogen atmosphere resulted in an increase in this nitrogen fraction, which accounted for the slight protein hydrolysis as well as for the nitrogen lost from the sum of amide and ammonia nitrogen.

When the slices were transferred from nitrogen back to air, the "other nitrogen" fraction gained partly from the continued protein hydrolysis and partly from the nitrate and amide nitrogen fractions.

Summary

1. Experiments are reported in which the respiration and the nitrogen metabolism of whole and sliced roots of *Raphanus sativus Aegyptiacus* are

studied. The effect of alternating air and nitrogen atmospheres is also discussed.

2. Respiration of mature whole roots proceeds along a steady low level for prolonged periods. Slicing of the root results in rapid and irregular increase in the respiration rate for about 25 hours, after which the latter subsides until it reaches a steady level parallel to that of the intact root but slightly higher.

3. Transference of radish root slices from air to nitrogen results in a drop in the respiration to a steady level below that in air. On re-exposing the slices to air, their respiration rate increases suddenly to a level higher than the air-line rate and then subsides gradually until it reaches the air-line rate after about 48 hours. The relationship between aerobic and anaerobic respiration rates shows that the oxidative anabolism, which occurs during aerobic respiration, is much lower than that reported by BLACKMAN for apple fruits.

4. Very little change takes place in the various nitrogen fractions in whole mature radish roots. The high irregular respiration of the sliced roots is always accompanied by protein synthesis at the expense of both nitrate and "other nitrogen" fractions. Some soluble organic nitrogen is always recovered from the water in which the slices are floated, and its origin is discussed.

5. Under anaerobic conditions, nitrate reduction is inhibited.

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NITROGEN METABOLISM OF DETACHED CORN LEAVES IN DARKNESS AND IN LIGHT[1]

F. G. VIETS, JR., E. I. WHITEHEAD, AND A. L. MOXON

When corn seedlings absorb large amounts of ammonium nitrogen from complete nutrient solutions high in ammonium, soluble nitrogen compounds accumulate in the tissues of shoot and root but the true protein remains relatively constant (15). Ammonia does not accumulate rapidly in tissues of the corn plant until asparagine, glutamine, a-amino nitrogen and peptides have reached relatively high values. In order to determine the role of these compounds in protein catabolism, the changes in nitrogen composition of detached leaves maintained in darkness and in intermittent light and darkness were studied and the results are reported here.

Yarwood (20) has recently reviewed the subject of detached leaf culture. Similar studies have been made on leaves of rhubarb (14), barley (21), Kikuyu grass (17, 18), and sudan grass (18), and oats (17) cultured in continuous darkness, and tobacco (12) and runner bean leaves (2) in light and darkness. The results show in general that protein hydrolysis begins soon after the leaf is detached and that amino nitrogen, and either or both of the amides, glutamine and asparagine, accumulate. Whether this loss of protein on detachment of the leaf is due to lack of supply of some external factor or hormone or to some internal factor has been the object of speculation (1, 16, 13).

Materials and methods

Seventy vigorous and not fully mature corn leaves and their attached sheaths were selected on July 25 from plants averaging about 3 ft. high in a field of open-pollinated yellow dent corn grown in a fertile field on the North Station Farm. Leaves were taken from the second and third nodes above the soil surface by cutting the sheath just above the node.

Ten leaves selected at random were dried immediately for a control or initial sample; 30 leaves were chosen for a light series and 30 for a dark series. The light series was placed in 6 earthenware 2-gallon crocks (5 leaves per crock) with the leaf sheaths inserted through large holes in the covers. Each crock contained 4 liters of 0.005 N $CaSO_4$ and the leaf sheath dipped 3–4 inches into the solution. The light series was kept in a white-washed greenhouse with air temperatures varying from 70° to 90° C., and subject to the usual diurnal fluctuations of light intensity. The dark series was similar in all details except that they were kept in a dark room in a temperature range of about 70° to 85° C.

At intervals of 3, 6, and 9 days at approximately 3 P.M. ten leaves were chosen at random from the dark series and light series, respectively, for weights and subsequent determinations. Green weights were taken after blotting the excess solution from the sheath, and the leaves dried for $2\frac{1}{2}$ hours in a forced draft oven at 80° C. Glutamine in plant material is stable under these drying conditions (10). Dry weights were obtained and the tissue ground in a Wiley mill to pass a 60 mesh screen. All samples were stored in bottles until analyses could be made.

The analytical procedures used were essentially those used previously (15) except for the following modifications. Ammonia and glutamine- and asparagine-amide nitrogen were determined on extracts made by mixing 3 gm. dried ground tissue and 60 cc. distilled H_2O and heating with stirring for 10 minutes to 75° C., then chilling in a refrigerator and filtering through quantitative paper. Extracts for total soluble and Van Slyke a-amino nitrogen were made by extracting 10 gm. dry tissue with 200 cc. dist. H_2O, heating to 80° C., filtering while hot, and cooling. Three gm. dry trichloracetic acid was added per 100 cc. extract and filtered to give the extract used. Total soluble nitrogen was determined by the iron-reduction method of PUCHER et al. (8). Residual a-amino nitrogen was obtained by subtracting the total amide nitrogen from the Van Slyke a-amino nitrogen (15). Nitrate nitrogen was determined by extracting 2.5 gm. ground dry tissue with 200 cc. hot distilled water in a 250-cc. flask, cooling, clarifying with 10 cc. saturated neutral lead acetate, and filtering after making to volume. To duplicate 100-cc. aliquots of extract and 200 cc. distilled H_2O in a Kjeldahl flask, 25 cc. of 10% NaOH was added and the volume boiled down to about 150 cc. to remove ammonia and hydrolyzable amide nitrogen. When cool, 200 cc. of water and 3 gm. Devarda's alloy were added, the nitrate nitrogen reduced to NH_3-nitrogen and distilled into standard acid.[2] Total nitrogen was obtained by summing the true protein nitrogen and total soluble nitrogen.

Results

The weights and analytical data are presented in table I. The leaves of cultures 4 and 5 at time of harvest were brown and dehydrated along the margins and there was a considerable amount of necrotic tissue at the tips of the blades. In cultures 6 and 7, which were 3 days older, the necrotic areas were more extensive and small brown patches of brown tissue occurred in the blade. A general yellowing of the leaf tissue did not occur, however, in either the dark or light cultures. The pattern of necrosis of leaf tissue was quite similar in both the dark and light series and is comparable to the injury obtained when ammonium nitrogen was supplied to intact plants from relatively concentrated solutions of $(NH_4)_2SO_4$ (15), except that rolling of the leaf blade did not occur.

Necrosis of the leaf tissue may account for the gradual decrease in water

[2] Essentially the method of R. W. GERDEL as modified by O. E. OLSON. Proc. South Dakota Acad. Sci. 20: 95–101. 1940.

TABLE I

WEIGHTS, PERCENTAGE MOISTURE, SUGAR CONTENT AND NITROGEN COMPONENTS OF DETACHED CORN LEAVES WITH SHEATHS CULTURED IN DILUTE CaSO$_4$ IN LIGHT AND IN CONTINUOUS DARKNESS AT THE END OF THE PERIODS INDICATED

	ORIGINAL	LIGHT			DARK		
Culture No.	1	2	4	6	3	5	7
Days	0	3	6	9	3	6	9
Green Weight, gm.	279	253	261	240	243	251	195
Dry Weight, gm.	33	38	44	47	36	37	37
% Moisture	88	85	83	80	85	85	81
Reducing sugars, % dry wt.	6.25	6.20	7.50	6.04	4.84	3.54	2.01
Sucrose, "	2.77	6.20	3.90	3.96	1.41	1.29	1.11
Total sugars,	9.02	12.4	11.4	10.0	6.25	4.83	3.12
Ammonia, mgm. N/kg. dry wt.	95	86	101	166	113	267	315
Glutamine, "	82	442	716	776	402	2,066	2,150
Asparagine, "	282	1,018	1,662	2,932	1,818	2,440	2,640
Residual α-amino, "	1,000	2,400	4,000	5,700	4,000	5,100	5,600
Nitrate, "	4,980	4,330	4,300	3,580	4,700	5,980	5,080
Total soluble, "	9,060	11,500	14,700	16,040	13,540	18,600	18,900
"True" protein, "	17,900	13,200	11,100	9,900	13,400	11,750	10,450
Total, "	26,960	24,700	25,800	25,940	26,940	30,350	29,350

content of the leaves under both environmental conditions as shown in table 1. However, dehydration of non-necrotic areas of the leaf may have also been involved. Water content of the leaves may have reached some maximal value between 0 and 3 days that is not shown by the data. VICKERY et al. (12) found that detached leaves of tobacco imbibed water from water and glucose solutions and maintained their higher water content for more than 100 hours after detachment.

Although the cultures were not sterile, only culture 7 showed any evidence of gross contamination. In this case the lower one-half inch of the sheath was slimy from bacterial growth. This portion was removed, dried, weighed, and the weight included in the total dry weight. The tissue, however, was discarded.

In the light series, the total sugars and sucrose increased during the period of culture while the reducing sugars remained relatively constant. Detachment of the leaf from the plant apparently blocks translocation of carbohydrate, but does not seriously interfere, if at all, with photosynthesis (20). On the other hand, in the dark series, a great loss of both sucrose and reducing sugars occurred. These two series of detached leaves, then represent two distinct levels of carbohydrate supply in comparison to which nitrogen metabolism may be studied.

Due to probable variations in the original weights of the leaves of each culture, the dry weights at harvest (table I) are not reliable indices of changes in weight during the experiment but an increase in weight of the light series due to photosynthesis and a decrease in weight of the dark series due to respiration is quite probable. This inadequacy of the data renders calculations of totals for each constituent of the leaf unreliable.

Nitrogen changes can be evaluated in percentage of total nitrogen, however, if losses of nitrogen did not occur. Losses of gaseous ammonia were not detected as Yemm (21) found with detached barley leaves cultured in the dark. At the end of the experiment, all of the solutions for the light series were composited, filtered, and aliquots taken for analyses. The solutions contained a total of 50 mg. total nitrogen of which 92% was NH_3-nitrogen. Composited solutions from the dark series contained a total of 17 mg. nitrogen, but ammonia-nitrogen was not present. No nitrates or nitrites were detected in the residual cultures of either series. The maximum loss of 50 mg. nitrogen for 30 leaves in the light series is equivalent to an average loss of 16.7 mg. nitrogen per 10 leaves. Since the initial leaves contained a total of 890 mg. total nitrogen the loss to the culture amounted to 1.9% of the total nitrogen. This loss is negligible in relation to the total nitrogen and permits comparison of the nitrogen constituents of the different cultures in per cent of total nitrogen per kilogram (dry weight basis), since all values for each culture are subject to the same increase or decrease in the dry weight. There is an increasing amount of evidence that loss of nitrogen in unknown gaseous form may occur (13), but it is relatively quite small in relation to the total nitrogen. VICKERY

et al. (12, 14) found that the total nitrogen of detached rhubarb and tobacco leaves did not change within the limits of accuracy of their analytical methods.

Figure 1 shows the changes in nitrogen constituents in per cent of total nitrogen per kilogram dry weight for each culture in the light and dark series, respectively. The distance between two lines represents the percentage for that constitutent of the total nitrogen. In both series hydrolysis of protein nitrogen occurred at a rapid rate and tended to decrease with time so that at the end of 9 days the protein level was little more than half of the initial value. The greater hydrolysis in the dark than in light was consistent and may have been significant, but the oustanding conclusion is that light and a high carbohydrate level resulting from photosynthesis and interrupted translocation exerted no sparing action on the leaf protein. VICKERY et al. (12) obtained similar results with tobacco leaves.
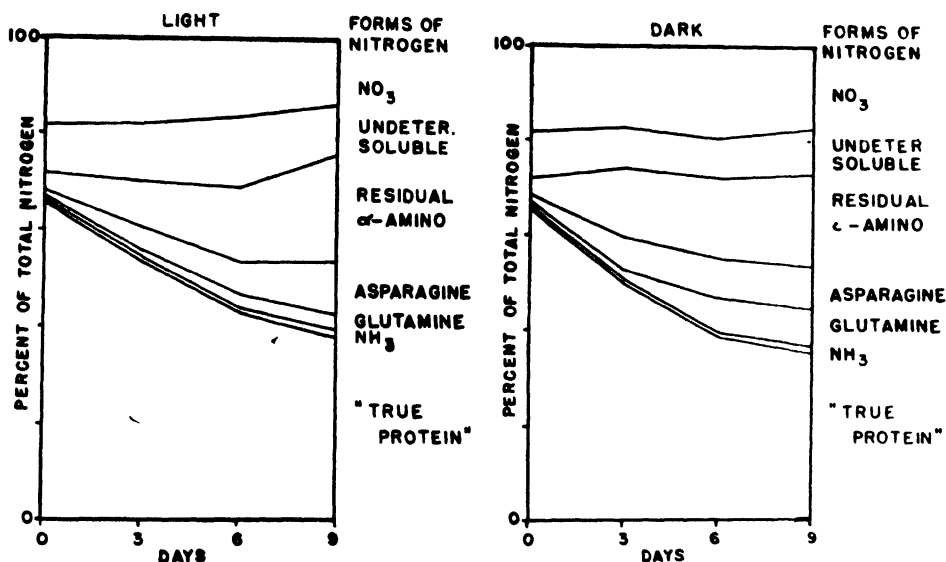
FIG. 1. Nitrogen distribution in detached corn leaves cultured for the period indicated in ordinary daylight and in complete darkness.

Soluble organic nitrogen compounds and ammonia accumulated in the leaf tissues of both series at the expense of the hydrolyzed protein, but some may have come in the light series from the reduction of nitrate and the subsequent combination of the reduction products with keto acids, etc. Amino nitrogen, asparagine, glutamine, and ammonium, in decreasing order of magnitudes, were the forms of nitrogen produced in the dark series. In the light series, undetermined soluble nitrogen accumulated in the early stages of protein hydrolysis, but the other constituents retained their same relative order, as in the dark series. Undetermined soluble nitrogen as here used consists of much peptide nitrogen (15). The relative rank of importance of these compounds or groups of compounds in protein catabolism of the leaf in light is quite comparable to their relative importance in metabolizing ammonia absorbed from the substrate by intact plants (15).

In the light series the accumulation of ammonia in the leaf tissues was less than in the dark series. If it is assumed, however, that the ammonia found in the residual cultures of the light series came from the tissues then calculations show that the amount of ammonium produced is not greatly different. In these experiments, like those previously reported on intact plants, there is no conclusive proof that accumulation of ammonia is responsible for the tissue injury noted since its accumulation is always associated with other profound metabolic changes.

Reduction of nitrates in the leaves of the light series may have occurred as indicated in figure 1. The original leaves were extremely high in nitrates, but this is not unusual for corn growing on soils in this section. The nitrates are not uniformly distributed in the leaf, but are higher in the sheath and the
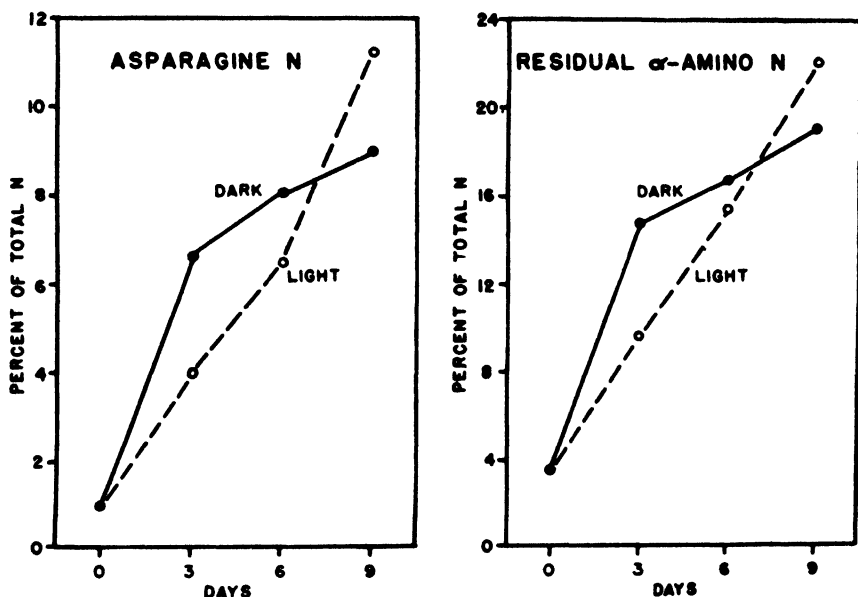
FIG. 2 (left). Asparagine accumulates rapidly in detached corn leaves cultured in either darkness or light.

FIG. 3 (right). Amino nitrogen, in addition to that associated with the amides, accumulates in detached corn leaves and at a rate twice that of asparagine nitrogen.

midrib than in the lamina. At the time of cutting, the leaf blade had a higher total nitrogen content, than the sheath tissue, about twice the a-amino and basic nitrogen content, approximately the same amounts of ammonia, glutamine, and asparagine and about one-half as much water-soluble nitrogen. In the dark series the nitrate content is somewhat erratic and in the interval 3 to 6 days the data indicate that nitrate actually accumulated. Whether this is significant cannot be determined from the data, but nitrate production has been noted in detached leaves cultured in the dark (5, 12).

Asparagine nitrogen and residual a-amino nitrogen in per cent of total nitrogen are plotted respectively in figures 2 and 3. Different units for the percentage scale are chosen in the graphs to show the similarity of the
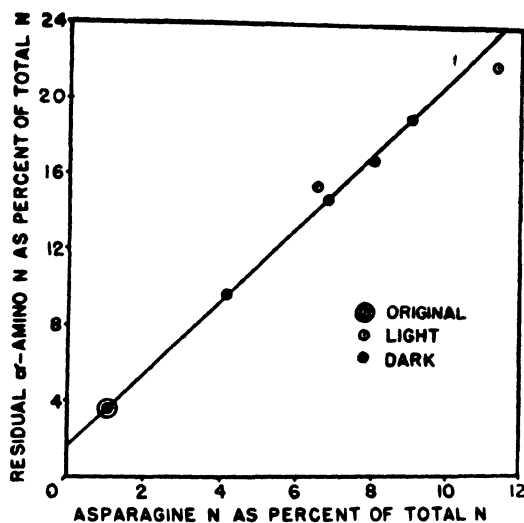
FIG. 4. Residual α-amino nitrogen accumulates at twice the rate of asparagine nitrogen in detached corn leaves regardless of the conditions of illumination.

asparagine and α-amino nitrogen curves in both the light series and in the dark series. This apparent equilibrium of asparagine and α-amino nitrogen, with the α-amino nitrogen twice that of the asparagine nitrogen, is more strikingly shown in figure 4 where asparagine nitrogen is plotted against residual α-amino nitrogen for both the dark and light series.

Figures 2 and 3 show that more asparagine and α-amino nitrogen were produced during the first 6 days of culture in darkness than in light and this may be related to the somewhat greater protein hydrolysis during this period in darkness than in light and to the fact that protein hydrolysis was more complete in darkness, peptides not accumulating as they did temporarily in the light series. At the end of 9 days, however, asparagine and α-amino nitrogen contents were greater in the light series than in the dark. This larger production of these constituents in the interval 6 to 9 days in the light series, as contrasted with the dark series, coincided with a significant decrease in the undetermined soluble or peptide nitrogen of the leaves of the light series.
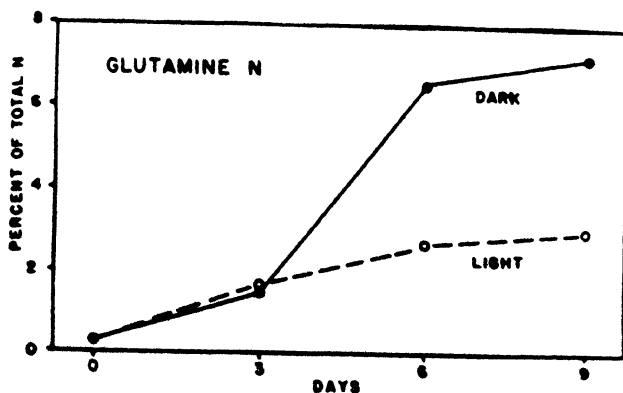
FIG. 5. Glutamine synthesis is favored by darkness in the later stages of culture of detached leaves.

Accumulation of glutamine is quite unlike that of asparagine and α-amino nitrogen as shown by figure 5 in comparison with figures 2 and 3. Glutamine production is much greater in the dark than in the light except in the first 3 days of culture. In the light asparagine and glutamine nitrogen increased 10.3% and 2.7% of the total nitrogen, respectively, in 9 days, whereas in the dark the gains were 8.0% and 7.0%, respectively.

In figure 6 glutamine nitrogen is plotted against residual α-amino nitrogen for both the dark and light series. Whereas a simple equilibrium exists between asparagine and residual α-amino nitrogen regardless of the light conditions and sugar content as shown in figure 4, the experimental conditions profoundly modify the glutamine α-amino nitrogen relationship. In
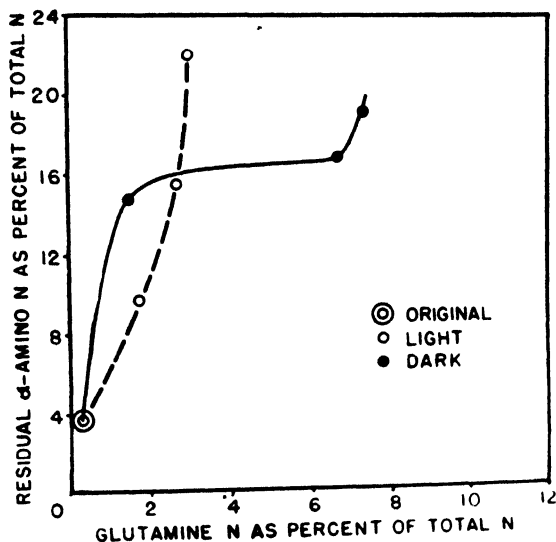
FIG. 6. Glutamine synthesis, unlike asparagine, is not related to residual amino nitrogen accumulation. Compare figure 4.

the light glutamine is unable to keep abreast of α-amino nitrogen accumulation, but in the dark from 6 to 9 days glutamine is preferentially formed.

Discussion

Three significant points are brought out by the data presented that are of fundamental importance to the physiologist and the biochemist in the study of plant metabolism.

The first is the similarity of the catabolic processes in the leaf following its detachment whether it is kept in light or darkness. In spite of an increasing carbohydrate supply in the illuminated leaf, the rate of protein hydrolysis and the accumulation of soluble nitrogen compounds, amino acids, and amides is quite similar to that of the starving leaf. Although detached leaves of some plants can form protein (7, 16), VICKERY et al. (12) found that tobacco leaves cultured in water, glucose and ammonium solutions lost protein at similar rates in continuous darkness or in continuous light. CHIBNALL (2) obtained similar results with cultures of runner bean leaves in

strongly diffused daylight and in darkness. He (1), later postulated hor-
monic control by the roots of the protein level of the leaf, but reserved judg-
ment until after further experimentation. WOOD (16) has recently dis-
cussed hormonic control and VICKERY et al. (13) have recently summarized
the problem as follows: "Thus detached leaf culture experiments furnish a
picture of the disintegration of the chemical systems of the cell when the
controls upon these delicately adjusted relationships are removed. The in-
ference is unavoidable that these controls play their roles effectively only
when the organism is intact; accordingly, certain factors in the controlling
mechanisms must have their origins in other tissues than the leaves."

PETRIE (6) regarded the protein level as being maintained at a super-
optimum concentration by oxidative metabolism with ample carbohydrate,
oxygen supply and assimilable nitrogen being essential for the continuous
supply of energy. If this picture is correct it is apparent with detached
corn leaves that detachment has interrupted the supply of some essential
component. To say that it is hormonic is only a convenience.

Studies with heavy nitrogen (4, 11) have shown that the protein of the
leaf is constantly undergoing hydrolysis and resynthesis. WOOD et al. (18)
have shown that some amino acids are preferentially oxidized in leaves.
Such preferential oxidation of amino acids in the protein cycle might lead to
a deficiency of an amino acid if it is synthesized only in the roots and trans-
located to the leaves. The importance of the roots of corn in the synthesis
of asparagine, glutamine and α-amino acids has been shown by the writers
(15). Further work with detached leaves cultured in dilute solutions of
single amino acids, vitamins, hormones, purines, pyrimidines, and extracts
of roots is needed.

The second point is the equilibrium existing between asparagine and
residual α-amino nitrogen over a wide range of concentrations and indepen-
dent of the conditions of hydrolysis as shown in figure 4. Such an equiva-
lence either in detached leaves or with intact plants has not been reported.
A change of 2% in residual amino nitrogen is equivalent to a change of 1%
in asparagine nitrogen.

CHIBNALL and NOLAN (3) have reported that leaf cytoplasmic protein
contains 7.44% amide nitrogen. Using this figure, the amount of amide
nitrogen expected from the hydrolysis of protein corrected for changes in
undetermined soluble nitrogen can be calculated and are shown in table II.
This assumes that the undetermined soluble nitrogen is largely peptide (15)
and of the same amino-acid composition as the hydrolyzed protein. The
equation used is: expected Δ-amide = 0.0744 (loss of protein + loss undeter-
mined soluble nitrogen). Table II also shows the actual changes in amide
nitrogen for various periods. Only about one-third of the amide nitrogen
synthesized can come from the protein hydrolyzed; the remainder is
synthesized in secondary reactions. Other investigations (12, 14, 21)
have shown with detached leaves that a large portion of the amides are of
secondary origin.

If the amides were of primary origin then a constant ratio of asparagine to residual α-amino nitrogen might be expected. However, since a large part of the asparagine is the product of oxidations of amino acids to produce ammonium, and the combination of this ammonia with aspartic acid, produced possibly in part by transaminations (9), the equivalence of amino nitrogen and asparagine cannot be explained with the data now available. This is more significant in view of the fact that probably not all of the amino acids represented in the determined complex enter into the equilibrium. Some are more easily oxidized than others (18), and some do not enter into transamination reactions (9).

The third point is the effect of darkness on the accumulation of glutamine. In the light cultures the rate of accumulation of glutamine decreased with time and did not keep pace with the accumulation of residual amino acids. In darkness, however, glutamine synthesis was very rapid in the 3–6

TABLE II

CALCULATED INCREASE IN AMIDE NITROGEN EXPECTED FROM PROTEIN AND PEPTIDES HYDROLYZED, THE INCREASE IN AMIDE OBTAINED, AND THEIR RATIO

PERIOD	AMIDE NITROGEN		
	CALCULATED	OBTAINED	OBTAINED/EXPECTED
Light	% of total N	% of total N	
0–3 days	0.72	2.28	3.2
0–6 days	1.33	3.94	2.96
0–9 days	2.01	6.48	3.22
Dark			
0–3 days	1.27	3.45	2.72
0–6 days	2.11	6.79	3.22
0–9 days	3.22	7.48	2.32

day period and at the end of 9 days they contained more than twice as much glutamine as the leaves in the light. Figure 6 shows no relationship between glutamine and residual amino nitrogen. Apparently metabolism conditioned by the presence or absence of light is of primary importance in the secondary synthesis of glutamine.

VICKERY et al. (12) found that light was essential to glutamine synthesis in cultured tobacco leaves, almost none being formed in leaves in the dark. Although these results with corn are contradictory to those with tobacco, they are not as clear cut because there was appreciable glutamine synthesis under both conditions.

Adequate explanation of the results obtained here cannot be given until more is known of the intermediates, precursors, and enzyme systems involved in bringing about the observed changes in composition.

Summary

1. Leaves with attached sheaths of field-grown dent corn were cultured in continuous darkness and in a greenhouse for periods up to 9 days, with

the sheaths dipping into dilute $CaSO_4$ solution. At 3-day intervals ten leaves from each series were removed, weighed, dried, reweighed and ground for determinations of sugars and various nitrogen fractions.

2. Reducing sugars and sucrose were rapidly depleted in the dark, but sucrose increased in the light series while reducing sugars were relatively constant.

3. In percentage of the total nitrogen, protein was hydrolyzed at comparable rates in both series. Residual α-amino acids, asparagine, glutamine and ammonia, in decreasing order of magnitude, accumulated in the leaves. In the early stages of protein hydrolysis in the light, undetermined soluble nitrogen, mostly peptides, accumulated, but was subsequently hydrolyzed.

4. Nitrates were reduced in the light series, but in the dark the results were erratic suggesting that nitrate formation might have been involved.

5. A linear relation between residual α-amino nitrogen and asparagine nitrogen in the leaves in both series was obtained in the ratio $2:1$.

6. Glutamine synthesis in the dark was much greater than in the light, and was not related to the amino acid level.

7. The similarity of the metabolic processes in light and dark is discussed in connection with the role of external factors in protein regulation in the leaf.

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STUDIES IN THE METABOLISM OF CRASSULACEAN PLANTS: THE BEHAVIOR OF EXCISED LEAVES OF *BRYOPHYLLUM CALYCINUM* DURING CULTURE IN WATER

GEORGE W. PUCHER, CHARLES S. LEAVENWORTH, WANDA D. GINTER, AND HUBERT BRADFORD VICKERY

(WITH TWELVE FIGURES)

A fundamental problem in experimental work with living tissues is the selection of samples in such a way that differences in composition which result from the treatment to which the material is exposed shall represent the effects of the treatment alone. There should be no opportunity for other processes to intervene and obscure the results. Complete isolation of the system under study can be effected, in the case of leaf tissue, only by excision from the plant. This has the disadvantage that the leaves are then merely "surviving organs" and soon begin to undergo changes that are ultimately lethal. Nevertheless, for a reasonable length of time, such leaves can be assumed to behave in a manner that reflects the normal course of metabolic events.

The technique of excised leaf culture as applied in this laboratory (4, 5) consists in the selection at random of a group of approximately equal samples from a quantity of leaves picked from the plants at the same time, and therefore, presumably, in the same biological and chemical condition. One or more samples are at once prepared for analysis, in order to provide a point of departure for the series, and the others are subjected to treatment. Samples are subsequently withdrawn from time to time for analysis, the data obtained being computed in terms of equal quantities of fresh tissue as weighed at the start of the experiment. Biological variation is minimized by the use of moderately large numbers of leaves per sample. Although the results are expressed in concentration units, they refer to originally equal and similar lots of tissue. Accordingly, the differences from sample to sample furnish, within the limitations of biological and statistical variation, a measure of the actual magnitudes of the changes that have occurred. Errors arising from translocation are eliminated and, presumably, the only phenomena that affect the data are those associated with photosynthesis, respiration, absorption of culture solution, transpiration, and chemical transformations occurring within the cells.

The diurnal variation of acidity characteristic of crassulacean plants is a phenomenon admirably suited for study by this general method. The entire cycle of changes is normally completed within 24 hours, a period sufficiently short to justify the assumption that irreversible catabolic changes which may be initiated are of minor significance in comparison with the

relatively much larger chemical changes associated with the normal metabolism of organic acids and carbohydrates. Accordingly, in the present paper, three experiments are described in which excised leaves of *Bryophyllum calycinum* were subjected to culture in water. In the first, leaves collected in the afternoon, at the time of low acidity, were cultured for 24 hours in the greenhouse exposed to the normal variation in illumination; in the second, similar leaves were cultured in complete darkness for 2 days and, in the third, leaves collected in the early morning, at the time of high acidity, were cultured in complete darkness also for 2 days.

Methods

PREPARATION OF SAMPLES

On March 12, 1940, 9 Bryophyllum plants from the lot described in a previous paper (3) were selected, and the compound leaves, beginning at the second from the top, were removed from 5 of them at 4 P.M. The individual leaflets were cut from the petioles and rapidly sorted into a number of piles according to size. The samples for the experiment were then chosen so that the same number of leaflets from each pile was included in each, there being three samples of 33 leaflets taken for one experiment and four samples of 34 leaflets for the second. One sample from each group was immediately placed in the drying oven at 80° C.

The samples to be subjected to culture were arranged in long V-shaped troughs with the bases of the leaflets immersed in water, the blades being supported without overlap by the walls of the troughs. The troughs containing the 33-leaflet samples were placed in the greenhouse, the others in a completely dark room. All samples were in position within half an hour. The temperature of the greenhouse ranged from 20 to 27° C. during the 24-hour period of the experiment, that of the dark room from 21 to 23°.

The following morning at 7, shortly after sunrise, the other 4 plants were treated in the same way, four 39-leaflet samples being taken of which three were set up in the dark room by 7:15, the fourth being at once dried. The experiment carried out in the greenhouse was continued for 24 hours; the two experiments in darkness were extended for a second day in order to obtain information on the initiation of catabolic changes.

The process of selection of samples provided for randomization within each sample with respect to the plant of origin. The plants chosen for the afternoon samples corresponded in size with the larger plants described in a previous paper (3), those chosen for the morning samples corresponded to the smaller plants. Fundamental data on all of the samples are shown in table I. Analytical results obtained as percentages of the crude dry weight were calculated, with the use of factors derived from these figures, so as to give the quantity of the component that would have been obtained if each sample of fresh leaf had weighed exactly 1000 grams at the start.

The limits within which the samples duplicated each other with respect to composition may best be illustrated by the data for total nitrogen and ash.

Regardless of metabolic transformations within the cells during the period of culture, there should be no significant loss of nitrogen nor of inorganic components from the samples and no gain from the water used as culture medium. The mean value for the total nitrogen of the 11 samples was 1.747 ± 0.072 gms. per kilo of original fresh weight. The standard deviation is ± 4.1% of the mean. The mean value for the ash was 8.94 ± 0.020 gm. per kilo, the deviation being ± 2.3% of the mean. Accordingly, it may be assumed that differences substantially greater than ± 4% observed for other components represent significant alterations in composition as a result of the treatment.

TABLE I

FUNDAMENTAL DATA ON SAMPLES OF EXCISED *Bryophyllum calycinum* LEAFLETS SUBJECTED TO CULTURE IN WATER. FIGURES NOT OTHERWISE DESIGNATED ARE GRAMS. THE TIMES ARE STANDARD TIME

TIME OF SAMPLING		ELAPSED TIME	NUMBER OF LEAFLETS	FRESH WEIGHT: START	FRESH WEIGHT: END	CRUDE DRY WEIGHT	CRUDE DRY WEIGHT PER KILO	FRESH WEIGHT PER KILO: END
		hrs.		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
ALTERNATE DARKNESS AND LIGHT								
4	P.M.	0	33	193.1		18.2	94.26	.
6	A.M.	14.0	33	182.5	185.6	19.0	104.1	1017
4	P.M.	24.0	33	180.0	177.5	18.9	105.0	986
DARKNESS: STARTING IN AFTERNOON								
4: 30 P.M.		0	34	231.3		21.3	92.08	
6: 30 A.M.		14.0	34	217.6	219.9	21.2	97.4	1010
4: 30 P.M.		24.0	34	219.8	221.9	21.2	96.45	1010
4	P.M.	47.5	34	202.3	201.5	18.7	92.43	996
DARKNESS: STARTING AT DAYBREAK								
7	A.M.	0	39	249.4		23.7	95.01	
4: 15 P.M.		9.25	39	243.5	252.3	23.1	94.86	1036
8	A.M.	25.0	39	250.5	257.3	21.7	86.64	1027
8	A.M.	49.0	39	251.1	247.0	21.5	85.61	983.5

Results

CULTURE IN ALTERNATE DARKNESS AND LIGHT

That diurnal variation in acidity occurs in detached leaves of Bryophyllum subjected to culture in water is evident from the curve for pH in figure 1. During the night, the pH dropped by 1.3 units, owing to synthesis of acids, and rose again during the day to a value even higher than that observed initially. The change was about twice as great (in pH units) as that observed in the leaves of intact plants described in a previous paper (3). These observations alone are sufficient to justify the fundamental assumption on which the present experiments are based, namely, that excision of the leaves does not seriously interfere with the normal metabolic functions for at least 24 hours.

Notwithstanding the large change in total acid reflected in this change in pH, the alkalinity of the ash (fig. 1) remained constant. The mean value for the alkalinity of the ash of the entire set of 11 samples was 195.1 ± 5.8 milliequivalents per kilo, the deviation being ± 3% of the mean. This is the same order of magnitude as the deviation in the nitrogen and ash content and is clearly a measure of the reproducibility of the samples rather than of any effect of treatment. The alkalinity of the ash is a determination of the difference between the number of equivalents of inorganic acidic and of inorganic basic components present after ignition of the tissue; inasmuch as no opportunity was afforded for translocation of inorganic acid or base into or out of the leaves, there was no way in which this difference could be altered by the treatment to which the leaves were subjected.

Organic solids apparently increased during the period of darkness and remained essentially constant during the day (fig. 1). However, the analytical determination of organic solids is especially difficult and subject to error and it is by no means certain, in view of the small number of samples, that the apparent increase, although it amounted to about 10%, was real. The similar samples cultured in the dark room (fig. 5) showed no comparable effect. The ash (fig. 1) and the total nitrogen (fig. 2), as has already been pointed out, showed no significant change in the course of the experiment.

The data presented so far are thus in agreement with the view that the three samples used for this experiment were initially sufficiently alike in composition to warrant the conclusion that changes in excess of 10% with respect to any analytical component represent alterations in composition as a result of the treatment to which they were exposed. The change in protein nitrogen (fig. 2) furnishes an illustration of this; during the night, the protein nitrogen increased from 0.61 to 1.06 gm. per kilo and then diminished during the day to 0.65 gm. The change was similar both in magnitude and in its relationship to light to that observed in leaves of intact plants subjected to analogous treatment (3). Furthermore, as in the previous experiment, there were changes in soluble organic nitrogen symmetrical with those of protein nitrogen, the one increasing when the other diminished and vice versa.

That the change in organic soluble nitrogen should be roughly equal and opposite to the change in protein nitrogen is to be expected. The quantity denoted protein nitrogen represents the nitrogen of the dried leaf tissue that remains insoluble after a sample is exhaustively extracted with hot 70% alcohol, a procedure designed to remove chlorophyll and other pigments together with the soluble carbohydrates and simpler nitrogenous components, and subsequently further extracted with boiling water to remove a small additional quantity of water-soluble nitrogenous material. It is assumed that nitrogen that remains insoluble under these conditions belongs exclusively to denatured protein. Wide application of the method has hitherto revealed no case that throws doubt on this conclusion. The soluble organic
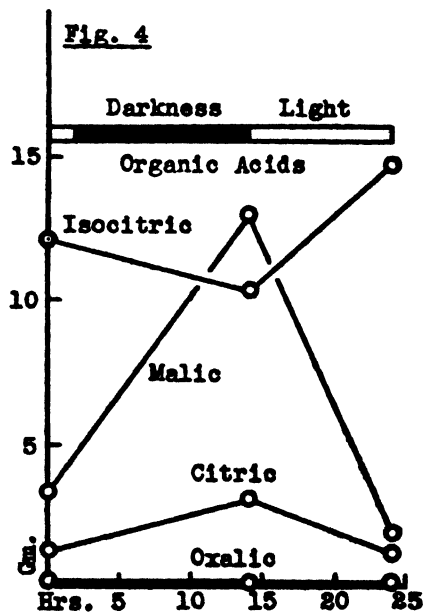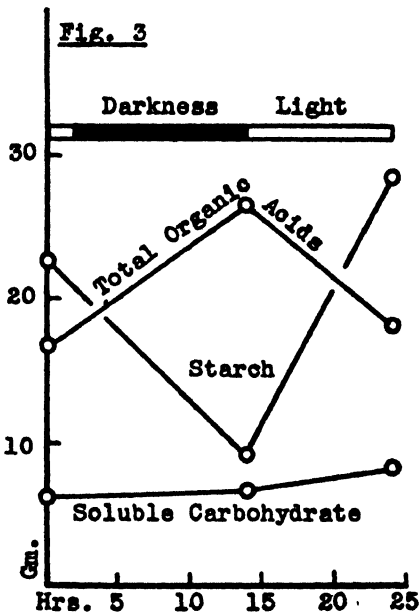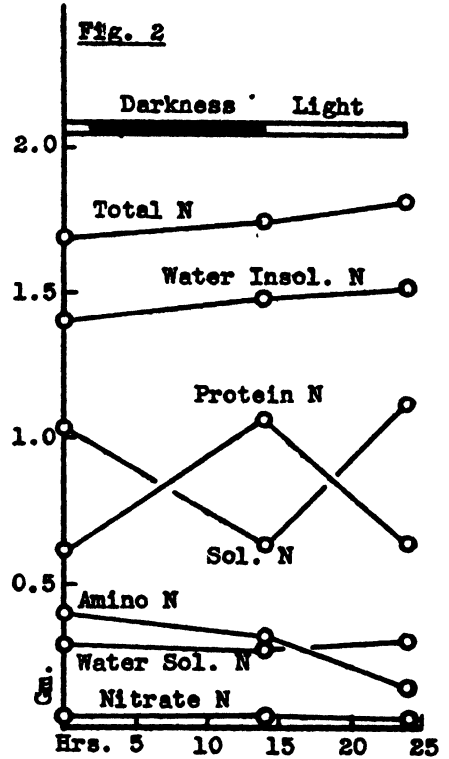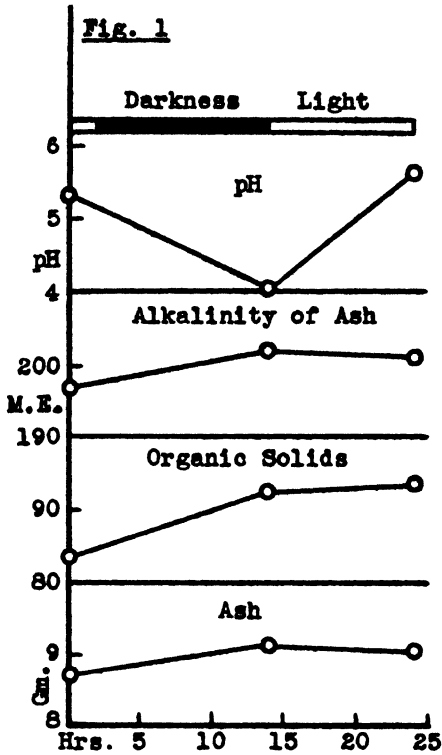
FIGS. 1 to 4. Composition of excised leaflets of *Bryophyllum calycinum* cultured in water for 24 hours in alternate darkness and light. Data are expressed in grams per kilo of fresh weight.

nitrogen is obtained by subtracting the protein nitrogen from the total nitrogen and correcting the difference for nitrate and for the trace of free ammonia nitrogen present.   If these are constant, as was very nearly the case in the present samples, the curve for soluble nitrogen must be nearly symmetrical with that for protein nitrogen.

Determinations were also made of the quantity of nitrogen that remained insoluble when samples of the dry tissue were extracted with hot water. With leaf tissues such as tobacco, results so obtained are practically the same as those after extraction successively with hot alcohol and hot water.   With Bryophyllum leaves, the quantity of water-soluble nitrogen (fig. 2) is much smaller than that of soluble organic nitrogen as usually determined, and the difference between the two curves (fig. 2) furnishes a measure of the additional nitrogen extracted by hot alcohol.   Correspondingly, the quantity of water-insoluble nitrogen (fig. 2) is much greater than the protein nitrogen. Neither curve shows any clear effect of the treatment to which the leaves were subjected.   Bryophyllum leaves obviously contain nitrogenous substances that possess solubility relationships quite unlike those of the proteins of such more thoroughly studied leaves as tobacco, and the interpretation of the present data is correspondingly uncertain.   If the method usually employed in this laboratory for the determination of protein nitrogen is in fact applicable to Bryophyllum, the data imply that a substantial part of the protein present in the early morning is converted, under the influence of light, into products that are soluble in hot alcohol.   During the night, these products are reconverted into material, presumably protein, that has the same solubility as before.   That the alteration in solubility is not merely an effect of the changed pH of the leaves was established by suitable separate experiments.

The question of the nature of the products that are soluble in hot alcohol remains for more detailed study.   That they are not normal products of proteolytic action such as amino acids or simple peptides is evident from the curve for amino nitrogen (fig. 2) for, at the time the protein nitrogen was increasing by substantially 0.4 gm., the amino nitrogen decreased by less than 0.1 gm.; correspondingly, while the protein nitrogen was diminishing by 0.35 gm. the amino nitrogen also diminished by 0.17 gm. instead of increasing.   The failure of these analytical quantities to correspond with each other either in magnitude or in the anticipated direction of change is evidence that the diurnal alteration in the apparent solubility of the protein is something other than protein digestion such as is observed in tobacco leaves cultured under similar conditions.

'   Diurnal variation of the total organic acids is shown in figure 3.   During the night, the acids increased from 16.9 to 26.7 gm. per kilo, an increase of 58% of the evening value; during the following day, they decreased to 18.1 gm.   As has already been shown by the curve for pH, the normal diurnal process of organic acid metabolism characteristic of this species was in no detectable way interfered with by excision of the leaves.   Corresponding in

point of time with the change in the acids, there was an even more extensive change, although in the opposite sense, in the quantity of starch. Starch decreased during the night from 22.6 to 9.2 gm. per kilo (calculated as glucose) and increased during the day to 28.5 gm. Nevertheless, the soluble carbohydrates scarcely changed significantly; there was at most a slight increase during the day.

The chemical possibilities presented by these changes in composition are far too complex for detailed interpretation, and all that can be attempted is to point out certain inferences that may justifiably be drawn. The quantities of starch and organic acids involved are such that a roughly quantitative relationship of the nature of an equilibrium between starch and acid might be assumed to exist, that is to say, a reaction analogous to that offered as a speculation by BENNET-CLARK (1) to account for the chief chemical reactions of crassulacean metabolism. During the night, 13.4 gm. of starch disappeared while 9.8 gm. of organic acids were formed. There was no change in the soluble carbohydrates so that the net change represents a loss of 3.6 gm. of organic substance. No such loss appears on the curve for organic solids; rather, there was an apparent increase of about 9 gm. which, from other considerations, it seems more conservative at present to interpret as being possibly due to biological variation between samples. Thus the evidence, as far as it goes, suggests that the respective alterations in the quantities of acids and of starch were the result of intracellular chemical reactions and it is quite possible that the acids arose in the course of a series of oxidation reactions which consumed starch.

During the day, however, 19.3 gm. of starch appeared and 8.6 gm. of acids disappeared. The net increase in organic solids from these two reactions is about 12 gm. since there was also an increase of about 1 gm. of soluble carbohydrate. However, no increase of this magnitude is shown by the curve for the organic solids (fig. 1); these in fact remained constant and it seems reasonable to assume that, if about 12 gm. of starch were newly formed by photosynthesis from carbon dioxide acquired from the air, there would have been a clearly evident rise of the curve in figure 1. Thus the formation of starch in light must, in this case, have also been largely the result of intracellular reactions and the quantity is considerably greater than could possibly have arisen from the organic acids even on the most favorable assumptions as to the efficiency of the reversal of the equilibrium which may have operated during the night.

By far the greater part of the change in total organic acids arose from the change in malic acid (fig. 4). This substance increased from 3.3 to 13.0 gm. per kilo, or by a factor of 4 during the night and decreased to less than the original value during the day. Citric acid underwent a parallel series of changes increasing from 1.3 to 3.1 gm. per kilo during the night and falling again during the day to 1.2 gm. Isocitric acid, however, behaved quite differently; it decreased from 12.1 to 10.4 gm. per kilo during the night and increased to 14.8 gm. during the day. These changes are in the opposite

sense to those of the malic and citric acids and they are proportionally much smaller ones; isocitric acid does not respond to illumination as extensively as the other two acids. The small quantity of oxalic acid present showed no significant change as a result of the treatment of the samples.

CULTURE IN COMPLETE DARKNESS

The data for the experiments carried out for 2 days in complete darkness are plotted in separate figures placed side by side for more convenient comparison of the effects upon leaves picked in the one case in the afternoon at the time of low acidity and, in the other, in the morning at the time of high acidity, these leaves, of course, having been in darkness during the night.
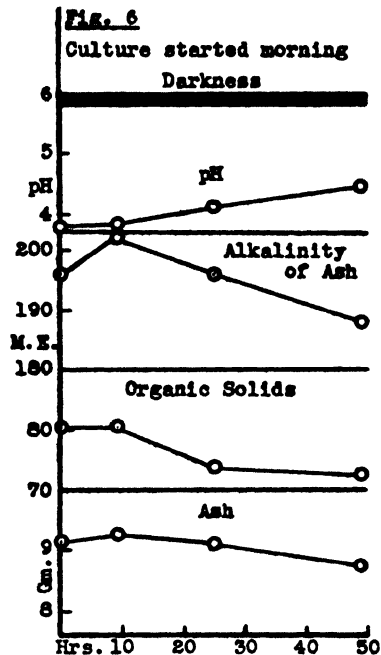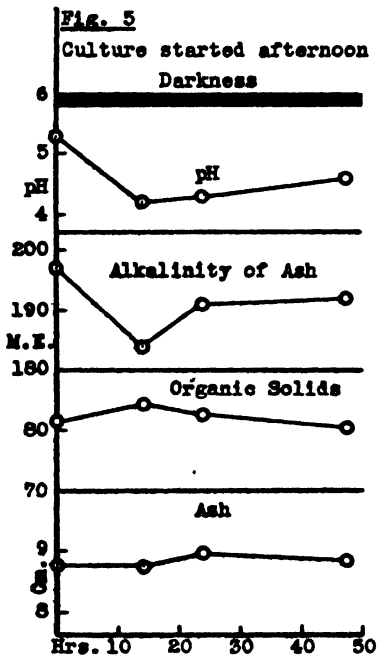
FIGS. 5, 6. Composition of excised leaflets of *Bryophyllum calycinum* cultured in water for 2 days in complete darkness. Data are expressed in grams per kilo of fresh weight. Figure 5 refers to leaves started in the afternoon at a time of low acidity; figure 6 to leaves started in the morning at a time of high acidity.

The pH of the samples placed on experiment in the afternoon was high (fig. 5) and dropped for the first few hours in the same way as it did in the first experiment. It then began slowly to rise and had increased by 0.4 units at the end of 2 days. The pH of the samples started in the morning was low (fig. 6) and there was a slow and steady rise over the 2-day period that totaled about 0.6 units. The early parts of these curves reflect metabolic changes that were probably not greatly different from those that occur in normal leaves; the events that took place during the second day, however, doubtless reflect the behavior of cells placed under gradually mounting metabolic stress. One of the first demonstrable effects of this stress is clearly a diminution in the acidity of the leaves.

The curves for alkalinity of ash, organic solids, and ash (figs. 5 and 6) and for total nitrogen (figs. 7 and 8) show, as did the similar data for the first experiment, no significant effect of the treatment of the samples. It is quite likely that the small drop in organic solids at the end of these two experiments represents a loss of organic material from respiration but the samples were too few in number and the experiment was not sufficiently prolonged to make this certain. Analogous experiments with tobacco leaves (4) and rhubarb leaves (5), however, have shown that such a result is to be anticipated.

The behavior of protein nitrogen in the two experiments was entirely different (figs. 7 and 8); in the samples started in the afternoon at a low
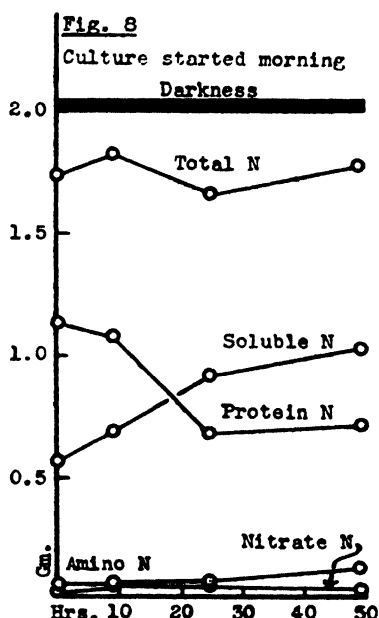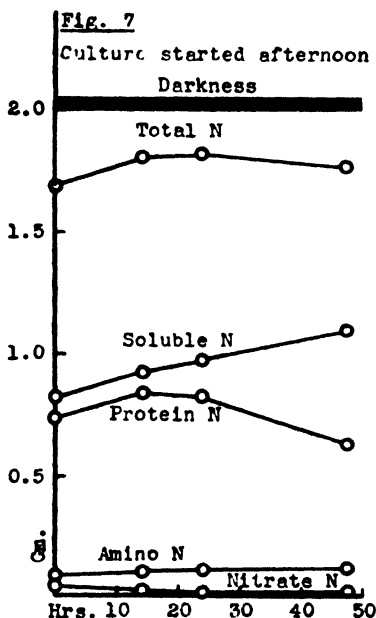
FIGS. 7, 8. Composition of excised leaflets of *Bryophyllum calycinum* cultured in water for 2 days in complete darkness. Data are expressed in grams per kilo of fresh weight. Figure 7 refers to leaves started in the afternoon at a time of low acidity; figure 8 to leaves started in the morning at a time of high acidity.

level, protein at first increased. After passing through a flat maximum, the quantity of protein nitrogen then decreased during the second day of the experiment. However, neither change was as striking as those encountered in the first experiment (fig. 2); the increase was about 13% of the afternoon value and was thus probably significant, the decrease was 25% of the maximum value and of the validity of this there can be little doubt.

The samples started in the morning at a high level of protein decreased only slightly in protein content during the first 9 hours but had dropped by 39% at the end of 24 hours. There was no significant change during the second day. The effect of culture in darkness on the protein of Bryophyllum leaves is thus dependent upon the level of the protein present in the

leaves at the beginning of the experiment.   If the culture is started at a low level at the end of the day, there appears to be a small increase, but if it is started in the morning at a high level, there is little if any significant change for about 9 hours; then, however, a change analogous to that observed in light supervenes and there is a marked fall.   Interpretation of these changes must await more detailed analytical studies; for the present, all that can be stated is that the fall in protein is not accompanied by a marked rise in amino nitrogen (fig. 8) as would be expected if the reaction were one of protein hydrolysis.   Rather, it would appear to be one of change in solubility of a substantial part of the protein such as might be supposed to result from some form of disaggregation into units that are not rendered insoluble by hot alcohol.

The failure of the curves for soluble nitrogen to follow courses exactly symmetrical with those for the protein arises in part from the minor irregularities in the curves for total nitrogen.   The changes that occurred in the early phases of the culture started in the afternoon were small and were about at the limit of certain detection by the analytical techniques.

The leaves placed on experiment in the afternoon at low acidity promptly increased in total organic acids (fig. 9) just as in the first experiment.   The acids remained at a high concentration for the whole of the first day but then rapidly diminished to a level lower than that at the start.   Comparison of the curve with that for pH (fig. 5) shows that the drop in total acids was not accompanied by a correspondingly large increase in the pH and it is evident that pH by itself is an unreliable index of the magnitude of such changes.   On the other hand, the leaves started in the morning at high acidity (fig. 10) did not maintain the level of organic acids then present; a continuous loss occurred although this did not become immediately evident on the curve for pH (fig. 6).

The behavior of the starch was surprising; in the leaves started in the afternoon (fig. 9), starch decreased during the period that the acids were increasing, remained constant while the acids were maintained at a high level but then *increased* during the period that the acids were falling in concentration.   This represents synthesis of starch in leaves in total darkness, an unusual observation at best, and one that might well be attributed to error had it not been confirmed by an even more striking synthesis of starch in the leaves started in the morning (fig. 10).   In these, starch increased from 6.2 to 12.6 gm. over the entire 2-day period at a rate which followed a smooth curve essentially symmetrical with that for the total acids.

It will be useful to consider the relative quantities of starch and organic acids involved in these changes.   Figure 9 shows that, during the first 14 hours in culture in darkness, 13.4 gm. of starch disappeared while 8.9 gm. of organic acids were formed.   The change in soluble carbohydrate was insignificant being only .0.5 gm.; thus it is possible that the organic acids that were formed arose from oxidation reactions in which starch was consumed.   There was a net decrease of 4.5 gm. of organic material, too little
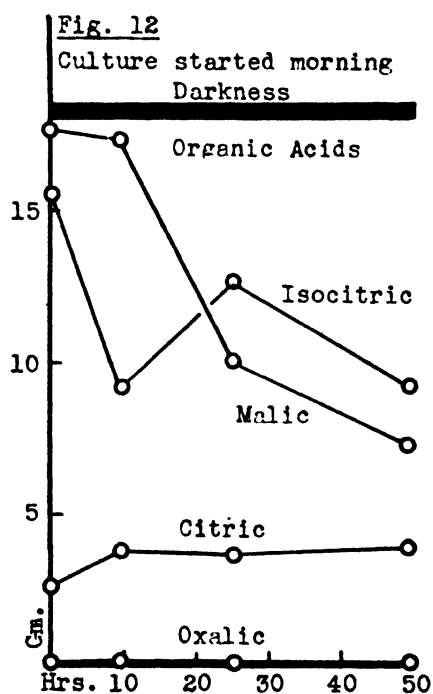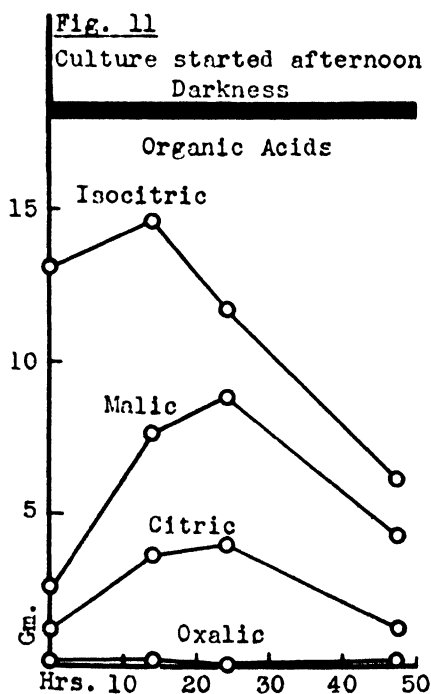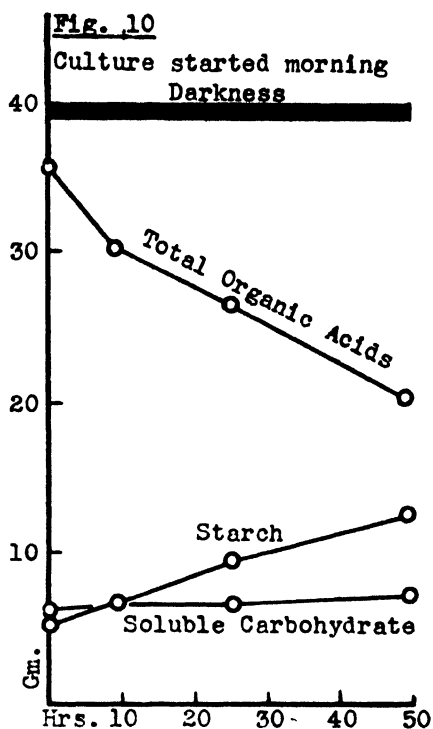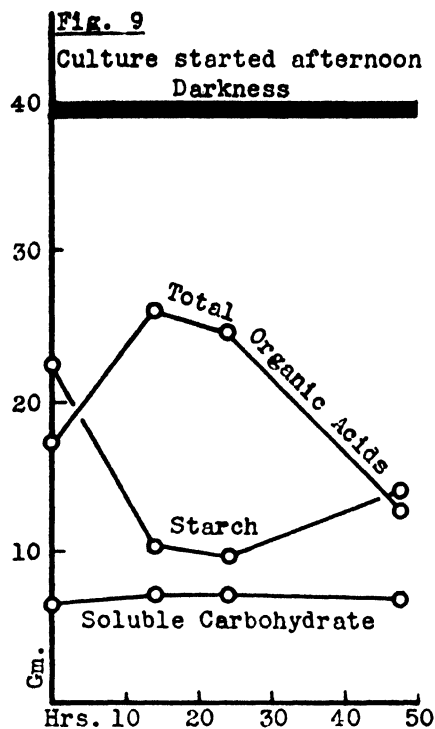
FIGS. 9 to 12. Composition of excised leaflets of *Bryophyllum calycinum* cultured in water for 2 days in complete darkness. Data are expressed in grams per kilo of fresh weight. Figures 9 and 11 refer to leaves started in the afternoon at a time of low acidity; figures 10 and 12 to leaves started in the morning at a time of high acidity.

to be revealed with certainty on the curve for organic solids (fig. 5) in any case even if these were the only chemical reactions that occurred. Actually, the organic solids in the 14-hour sample were slightly greater than those in the initial sample although the difference was well within the limits of uncertainty in the determination.

During the second day of the culture period, organic acids diminished by 11.7 gm. and starch increased by 4.3 gm. Compared with the experiment carried out in daylight, this is a small increase in starch. It was inferred from the data from the experiment in daylight that the organic acids could have contributed at most only a part of the material from which the starch was synthesized during illumination even on the assumption of complete reversibility of the reactions whereby the acids may have been produced in darkness. In the present case, however, judging from the quantities alone, there is a possibility that all of the starch formed in darkness could have arisen from such a reversal. However, this is not proof that such reactions occurred; all that has been established is that a significant quantity of starch was formed in darkness during a period when a substantially larger quantity of organic acids disappeared.

In the experiment in which the leaves were high in organic acids at the start (i.e., leaves which had remained attached to the plants during the night and in which the normal reactions of darkness had taken place), 9.4 gm. of acids disappeared during the first day and 6.1 during the second. The quantities of starch formed in these intervals were, respectively, 3.1 and 3.2 gm. Thus the behavior throughout was analogous to that during the second day of the other experiment in darkness.

The details of the transformations of the organic acids are shown in figures 11 and 12. In the leaves started in the afternoon, there was a large increase of malic acid and a substantial one of citric acid during the first 24 hours. During this interval, isocitric acid first increased and then diminished. Throughout the second day, all three acids diminished at almost equal rates. In the leaves started in the morning, malic acid remained nearly constant for 9 hours and then diminished rapidly, citric acid increased slightly at first, while isocitric acid underwent a sharp drop followed by a rise. During the second day, both malic and isocitric acids diminished while citric acid remained essentially constant. The relationships among the three acids strongly suggest that interconversions may have occurred; the temporary maintenance of malic acid at the initial high level and the small production of citric acid may both be results of reactions that consumed isocitric acid. If malic acid was produced as rapidly from isocitric acid as it was decomposed in the course of other reactions, the net quantity present would remain unchanged. The increase in citric acid may be assumed to result from transformation of isocitric acid via aconitic acid, a reaction well known to occur in muscle tissue (2). In the interval between the ninth and twenty-fifth hours of the culture period, the sharp fall in malic acid and the corresponding increase in isocitric acid may have been the effect of a reversal of the reaction that occurred during the first 9 hours.

Discussion

The chemical changes that occur during the first 24 hours of culture of excised leaves of Bryophyllum are, qualitatively, closely similar to those observed in normal leaves (3). Marked fluctuations occur in the form of nitrogen that is insoluble in hot alcohol and hot water and which has been interpreted as representing protein nitrogen. Leaves that have recently been illuminated are low in this component but protein is rapidly formed in such leaves when they are placed in the dark. Corresponding changes in the opposite direction take place in the soluble nitrogen. The acidity, whether measured as pH or by the titration of the organic acids extracted from the tissue, likewise undergoes wide variations, acids being synthesized in darkness and decomposed in light. Starch is formed during illumination but, unless the organic acids are undergoing decomposition, disappears in darkness so that the fluctuations are in the opposite sense to those of the organic acids. The largest share in the changes in the organic acids is taken by malic acid; citric acid varies in the same direction as malic acid but through a smaller range. Isocitric acid, however, varies in a less regular manner and the changes that it undergoes are frequently in the opposite sense to those of malic acid, sometimes being large, sometimes small, in relation to it.

The chief technical advantage of the leaf culture method is that, because of the complete isolation of the biological system, inferences may be drawn from the quantities of substances involved in the reactions provided the changes are large enough to be outside the range of probable biological variation among samples. Within certain limits, therefore, specific interpretations of some of the reactions that occur may be attempted.

It will be useful to point out the contrasts between the behavior of Bryophyllum leaves and those of tobacco when subjected to culture. Excised tobacco leaves cultured in water in light (4) exhibit a prompt and surprisingly large increase in organic solids, for the most part because of the synthesis of soluble carbohydrates and starch. In darkness, they undergo equally prompt losses owing to the uncompensated effect of respiration, and these losses fall heavily upon the carbohydrates and malic acid. The protein nitrogen diminishes rapidly under both conditions of culture; evidence was secured that implied the complete destruction of that part of the protein that disappeared, and indicated that the simple nitrogenous products formed underwent a succession of complex transformations. The experiments with tobacco leaves were extended for several days so that there is little doubt that the greater part of the changes observed, especially in the later stages, were catabolic in nature. They showed, however, that leaf cells are the seat of intense metabolic activity, and many of the changes were initiated within the first 24 hours.

Excised Bryophyllum leaves likewise undergo profound metabolic changes in composition during culture but, in this species, some of the more conspicuous changes are a part of the normal cyclic behavior of the tissue

under the influence of diurnal variation in illumination.   Certain of the
reactions so clearly observed in tobacco leaves were, on the other hand,
scarcely if at all demonstrable in this species in part because of the brevity
of the experiment.   This statement applies in particular to the reactions
between the tissues and the oxygen and carbon dioxide of the air; no conclu-
sive evidence was secured of photosynthesis in terms of an increase of organic
solids in light, nor could loss of solids by respiration in darkness be demon-
strated although it is possible that experiments more prolonged than the
present ones might be successful in this.   The evidence, as far as it goes,
suggests what may be termed a more closed metabolic economy than that of
the tobacco leaf, and observations that bear on this aspect of the problem
are to be found in the literature.   The work of BENNET-CLARK (1) and of
WOLF (6) on the respiratory quotient, for example, shows that the liberation
of carbon dioxide drops to a very low level shortly after the leaves are placed
in darkness at the time that acid production increases in intensity.   It is
reasonable to infer that carbon dioxide is being utilized within the tissues
for the synthesis of organic acids, possibly, for example, of oxalacetic acid
from pyruvic acid by the Wood and Werkman reaction.   Later, during
continued culture in darkness (i.e., after about 12 hours), carbon dioxide
liberation by the leaves increases and this occurs at the time when the organic
acids pass their maximum and begin to drop in concentration.   Its liberation
is an evidence of decarboxylation reactions.

BENNET-CLARK likewise observed that oxygen uptake is high during the
early phase of acid production but later diminishes.   This is in agreement
with the assumption that the formation of organic acids is a result of oxida-
tion reactions involving carbohydrates, particularly starch.   It is to be noted
that both oxidation and carbon dioxide fixation are essential if the organic
acid metabolism of Bryophyllum leaves is to be accounted for in terms of
the tricarboxylic acid cycle of KREBS.   Pyruvic acid may be assumed to
arise from the decomposition of hexoses but an increase in length of the
three carbon chain of pyruvic acid to four carbon atoms (i.e., conversion to
oxalacetic acid) is essential if the subsequent production of malic acid is to
be accounted for.   Furthermore, oxalacetic acid is also an essential inter-
mediate for the formation of isocitric and citric acids.   Regardless, there-
fore, of the absence of direct information on the presence of the specific
enzyme systems necessary for these reactions, one is justified in advancing,
as an hypothesis, the view that the transformations of organic acids observed
in Bryophyllum leaves are an expression of the activity of such enzyme
systems.   On this view, the several organic acids are intimately linked with
each other by chemical equilibria and transformation of one acid into an-
other, of carbohydrates into organic acids and even the reverse transforma-
tion of organic acids to carbohydrates receive a rational explanation.   The
synthesis of starch in darkness when the concentration of organic acids is
high becomes a phenomenon that is to be anticipated.   It should be pointed
out that all of the analytical data of the present experiments are in agree-
ment with the requirements of this hypothesis.

During the second 24-hour period of the present experiments, however, other phenomena began to make their appearance. The early culture experiments of BENNET-CLARK showed that the organic acids of *Sedum praealtum* leaves passed through a maximum in concentration after about 12 hours in darkness and subsequently declined. At the same time, carbon dioxide production increased. WOLF has made somewhat similar observations on *Bryophyllum calycinum* leaves, and the phenomena are very likely general for the Crassulaceae. In the present experiments, certain reactions were initiated which were the reverse of those noted during the first day. It is probably correct to assume that such reactions represent the beginning of the catabolic processes that inevitably follow excision of the leaf. One of the first to be demonstrable is, as a rule, the transformation of the nitrogen of protein into forms that are soluble and, in particular, an increase in amino nitrogen presumably produced by proteolytic action. These effects are clearly evident in tobacco and rhubarb leaves subjected to culture under analogous conditions (**4, 5**). They are not especially marked in the present case, however, although the culture started in the afternoon does show them to a possibly significant extent (fig. 7).

Another effect well shown by tobacco and rhubarb leaves is the loss of organic solids from respiration but, again, the present experiments were not sufficiently prolonged for this to become unequivocally clear (figs. 5 and 6). However, the disappearance of organic acids, especially malic acid, with a corresponding increase in pH is invariably observed and the present experiments show this especially well. All three of the chief organic acid components decreased sharply during the second day of the experiment started in the afternoon, two of them followed the same course in the experiment started in the morning. This effect may be regarded as an evidence of the increasing metabolic stress to which the excised leaves were subjected. Even in this case, however, the analogy with tobacco and rhubarb leaves is not close. The Bryophyllum leaves became enriched in starch and there was no effect upon the soluble carbohydrates. In tobacco and rhubarb leaves, on the other hand, carbohydrates are early involved in the general picture of catabolism.

Taken together, then, the chemical evidence of the present experiments suggests a general metabolism adapted for survival under unfavorable conditions. The response, rather early in the period of stress, is to lay up a store of starch. It is quite possible that the well-known capacity of excised leaves of Bryophyllum to survive and reproduce by buds which develop at the margins may be the ultimate expression of such a provision.

Summary

Equal samples of leaflets excised from *Bryophyllum calycinum* plants were subjected to culture with the base of each leaflet immersed in water, one set of samples taken in the late afternoon being treated in the greenhouse and a second set in a completely dark room. A third set collected

early in the morning was likewise placed in the dark room.   Analytical data were computed in terms of 1 kilogram of fresh tissue weighed at the time of sampling.

There were no significant effects in any case upon the quantity of organic solids nor upon the ash, the alkalinity of the ash, or the total nitrogen; even the soluble carbohydrates failed to change.   The samples cultured in the greenhouse increased sharply in organic acids during the night and decreased during the day, the greater part of the change being due to malic acid with citric acid playing a smaller but similar role.   Isocitric acid decreased at night and increased during the day.

Starch varied in a manner the converse of organic acids, the fluctuations being in each case through a wider range than those of the acids.   Thus it is possible that organic acids synthesized at night arose from oxidation of carbohydrates.   However, some source of starch other than the organic acids must be invoked to account for the starch synthesized during the day, yet the failure of the organic solids to increase significantly indicates that photosynthesis played only a small part.

Protein increased during the night and decreased during the day, soluble nitrogen undergoing similar changes in the reverse direction.

The leaves cultured in darkness starting in the afternoon at low acidity increased in malic, citric, and isocitric acids for 14 hours.   The increase in malic and citric acids continued for the rest of the first day but isocitric acid then began to decrease.   All three acids decreased during the second day.

Starch decreased during the first day in darkness but increased significantly during the second.   Protein increased slightly for 14 hours and then decreased slowly.

The leaves cultured in darkness starting in the morning at high acidity began to lose isocitric acid at once.   Malic acid dropped slightly but citric acid increased; later malic acid dropped sharply, isocitric acid increased and citric acid remained constant.   During the second day, isocitric acid and malic acid decreased, and citric acid remained unchanged.

Starch increased slowly but continuously throughout the 2-day period in darkness.   Protein decreased during the first day and remained unchanged during the second.

The changes in organic acids and starch can be largely accounted for if it is assumed that the several substances are linked by enzymatic reactions in a system analogous to the Krebs tricarboxylic acid cycle.

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PLANT GROWTH WITH ARTIFICIAL SOURCES OF RADIANT ENERGY[1]

ALICE P. WITHROW AND ROBERT B. WITHROW

(WITH FIVE FIGURES)

Introduction

In the design of experiments where plants are to be grown under controlled radiation conditions, it is necessary to have information on the relative power efficiency and the type of growth obtainable with various lamp sources, for the proper selection of equipment. It is well known that the growth form of plants is dependent upon the incident irradiance, photoperiod, and the spectral characteristics of the radiant energy (1, 2, 11, 14, 15, 16).

Before the introduction of the gaseous discharge lamps, most of the studies were made using the incandescent tungsten filament lamp as a source (7, 9, 14). While the growth rate obtained with the bare incandescent lamp frequently was comparable with that often secured under greenhouse conditions when sufficiently high irradiances were used, the plants usually had thin stems and longer internodes than those grown with solar irradiation. With the advent of the modern type gaseous discharge lamps, ROODENBURG (17), ARTHUR and his co-workers (1, 2, 3), and others studied the growth obtainable with these sources.

ARTHUR and STEWART (1) observed production of buckwheat plants under incandescent, neon, mercury, and sodium vapor lamps. They found that, with equal irradiances as measured with a Weston illuminometer, the efficiency in production of dry matter decreased in the following order: neon, incandescent, sodium, and mercury. When the radiant energy was calculated on an equal energy basis, they concluded that the actual order of efficiency was sodium, neon, incandescent, and mercury. When sodium alone was tried (2), it was found that the plants did not grow well over long periods of time. Supplementing the sodium with two hours per day from an 85-watt capillary mercury vapor lamp, caused an improvement in growth with a number of varieties of plants.

MITCHELL (12) grew tomato plants, using a carbon arc and an incandescent filament lamp as sources. He found that total synthesis of dry matter was greater per unit of time under the incandescent lamp when equivalent energies, as balanced with a Weston cell, were used. The plants synthesized more dry matter, although the height was less, under the carbon arc when equal total radiant energies, as measured by a nonselective detector, were used.

JOHNSTON (11) grew tomato under varying combinations of water-filtered incandescent and mercury arc radiant energy and concluded from his studies that a greater amount of dry weight is produced with an incandescent lamp when it is enriched with blue from a mercury lamp to the extent of 14% to 51%.

NAYLOR and GERNER (13), ROHRBAUGH (16), and STOUTEMEYER and CLOSE (18) have published data on plants produced under fluorescent lamps, showing that satisfactory growth can be secured with this source. However, no comparative data for the fluorescent with other lamp sources have been published.

The most important radiation characteristics of lamps to be considered for use in growing plants are (1) spectral energy distribution; (2) proportion of input electrical energy dissipated as radiated infrared; and (3) efficiency of production of visible radiant energy within the region of about 4500 A to 7000 A. The proportion of electrical energy dissipated as radiated infrared sets a maximum limit on the visible irradiance which can be used with bare lamps. The infrared beyond 8000 A apparently has little effect other than to raise the temperature of the foliage. If the proportion of radiated energy in the infrared is high, as with the incandescent lamp, excessive heating of the leaves may occur at high irradiances unless suitable filters are interposed between the lamp and the plants.

The present experiments were designed to investigate the comparative growth responses of certain herbaceous annuals under several sources of artificial radiant energy, including the fluorescent, incandescent and high pressure mercury arc lamps. These lamp sources were selected because they represent the most practical types of equipment for general use on the bases of (1) commercial availability; (2) installation cost; (3) ease of lamp replacement and operation; and (4) power efficiency in terms of visible radiant energy production.

Radiation characteristics of lamps

The spectral characteristics and electrical power efficiency of lamp sources depend primarily on the type of radiant energy producing system used; i.e., incandescent filament, gaseous discharge, and/or phosphors. Secondary in importance are operating conditions such as lamp voltage, ambient temperature, age of lamp and type of reflector system. The spectral, electrical, and physical characteristics of commercially available lamp sources are thoroughly discussed in manufacturers' bulletins (8) and in engineering publications on lamp characteristics (4, 5, 6).

The energy distribution of the general service type of *tungsten filament incandescent lamp* is characterized by a continuous spectrum which has a maximum in the near infrared at about 10,000 A. The energy continuously decreases throughout the visible spectrum as the wavelengths become shorter and is practically zero at about 3200 A, which is the ultraviolet transmitting limit of ordinary glass. The incandescent lamp is a source which, compared

to solar radiant energy, is relatively rich in red and weak in blue radiant energy. A 500-watt incandescent lamp radiates beyond the bulb only 12% of the input watts into the visible spectrum and over 70% is radiated as infrared. Most of the remaining 18% is dissipated as heat to the surrounding air and to the base of the lamp by convection and conduction. The ultraviolet radiated beyond the bulb is less than 0.1% of the total input watts.

The luminous efficiency of incandescent lamps of from 500 to 3000 watts in the general service types varies from slightly less than 20 to nearly 30 lumens per watt. The lumen is a convenient unit of luminous flux and evaluates spectrally the radiant energy emitted by the lamp in terms of the sensitivity of the human eye. The footcandle is a similar unit of irradiance. In spite of serious limitations as discussed in a previous report (19), the lumen and the footcandle are still the most practical units of visible radiant flux and irradiance respectively. These units are useful, however, only in comparing sources of similar spectral distribution within the visible spectrum.

One of the principal advantages of the incandescent lamp for general use is that no auxiliary equipment is required for its operation. Therefore, the luminous efficiency figures given above represent overall efficiencies. The rated life for general service incandescent lamps in wattages from 500 to 1500 is 1000 hours.

The spectral energy distribution of the *high pressure mercury arc* in glass is characterized by the line spectrum of mercury, with a small proportion of the total radiated energy as a continuous spectrum. Most of the energy within the ultraviolet and visible spectrum is distributed within six groups of lines at 3341, 3654, 4047, 4358, 5461 A and the doublet centered about 5780 A. The mercury arc is one of the most efficient sources of blue radiant energy, but is extremely low in the red. Even for the glass lamps, from 1% to 2.3% of the input wattage is radiated as ultraviolet. For the 400-watt H–1 lamp, which was used in these experiments, the radiated infrared is less than that of the incandescent, but higher than the fluorescent.

The rated luminous efficiency of the 400-watt lamp alone is 40 lumens per watt. When account is taken of the 52 watts dissipated in a single lamp transformer, in addition to the wattage consumed by the lamp itself, the overall luminous efficiency is actually 35.4 lumens per watt. These lamps have a rated life of 3000 hours.

The *fluorescent lamp* is a type of low pressure mercury arc, in which the inside surface of the glass is coated with phosphors which transform the ultraviolet energy of the mercury line at 2537 A into radiant energy of longer wavelengths, which include the visible and ultraviolet. The fluorescent lamp phosphors emit a continuous spectrum, the energy distribution of which depends upon the choice of phosphor materials. The proportion of the input power radiated within the visible spectrum is a function of the nature of the phosphor, and length and diameter of the tube and other design and operating factors. For the 40-watt daylight fluorescent lamp, about

0.8% of the input watts is in the ultraviolet, 20.5% is radiated in the visible, and 26.5% is radiated as infrared. About 53% of the input energy is dissipated in convection and conduction. Thus most of the input energy is carried away to the surrounding air and is not available directly for raising the temperature of the foliage of plants placed under the lamps.

The overall luminous efficiency of the 30-watt white fluorescent lamp, which was used in these experiments, is 40.5 lumens per watt; for the daylight lamp, the efficiency is 37.2 lumens per watt. These figures are based on the use of 115-volt tulamp auxiliaries. The rated life of the fluorescent lamps, standard lamp series, is 2500 hours.

Experimental methods

The plants were grown in air-conditioned growth chambers. The air temperatures were controlled within $\pm 0.5°$ to $3°$ C. of the temperatures designated in the tables, depending on the lamp units. The humidity was kept in the range of 70% to 80%. Subirrigation gravel culture was used, with a double concentration of a Purdue F nutrient solution (**20**). Manganese was supplied weekly to the solution and iron three times weekly. No other micronutrients were added as previous experience had shown that the gravel was capable of supplying all of the other essential micronutrients. The pH was maintained between 5.5 and 6.5, except for soybean, in which case the range was 6.5 to 7.0. The nutrient solution was changed every two weeks. All of the plants received the same solution, regardless of the radiation treatments. This caused some difficulty because the nutritional requirements were found to be somewhat different for the plants growing under the various lamp sources.

Tungsten, fluorescent, and high pressure mercury arc (H–1) lamps were used as indicated in the tables. The footcandle measurements were made with a Weston photronic cell equipped with a green filter to correct its spectral sensitivity to that of the human eye. The measurements were checked with a Macbeth illuminometer. The footcandle (fc) values given are those at the gravel surface at the beginning of the experimental period. As the plants became taller, the values varied from the original ones given. Calculations for power consumption and total radiant energies were made from data presented by BARNES and his co-workers (**4, 5, 6, 8**). The number of hours of radiation differed in the various experiments and are shown in the tables. The radiation treatments with the sources were varied on the basis of equal power consumption, equivalent footcandles and equal total visible energy.

Results

EQUAL POWER CONSUMPTION

Nobel spinach and Heart of France variety of China aster were grown for 50 days under the radiation treatments designated in table I. The lamp sources were 1000-watt standard incandescent, 3500° K., 30-watt white fluo-

rescent and 400-watt H–1 high pressure mercury arc lamps. The power consumption was the same per square foot for the different sources. Under these conditions, the total visible radiant energy under the incandescent was 58% of that under the fluorescent and mercury lamps as calculated from the data of BARNES and FORSYTHE (**4, 5, 6, 8**). The lamps were on for 15 hours daily.

The largest and most vigorous plants of China aster, with the most profuse flowering, occurred with the incandescent radiant energy, with an intermediate growth and flowering response under the fluorescent. The plants under the mercury were small and averaged only one flower bud per plant.

TABLE I

GROWTH AND FLOWERING WITH EQUAL POWER CONSUMPTION

LAMP SOURCE*	PLANT MATERIAL									
	HEART OF FRANCE ASTER					NOBEL SPINACH				
	NO. PLANTS	AV. HT.	AV. DRY WT.	TOP-ROOT RATIO	AV. NO. FLOWER STALKS	NO. PLANTS	AV. HT.	AV. DRY WT.	TOP-ROOT RATIO	AV. NO. FLOWER STALKS
		cm.	gm.				mm.	gm.		
Incandescent 700 fc	12	46.9	8.1	9.6	9.5	20	5.2	1.4	14.4	0
Fluorescent, white 1200 fc	12	21.4	4.8	6.6	3.0	20	52.3	1.8	21.5	0.74
Mercury 1200 fc	12	18.6	3.8	7.7	1.0	20	14.7	0.6	25.0	0.18

* Same power consumption per square foot for all sources.

The spinach plants grown under the fluorescent were vigorous and dark green, and 74% had flower stalks. Those under the mercury were much less vigorous, with flower stalks on but 18% of the plants. With the incandescent lamp, spinach was small and had no flower stalks or even microscopic floral primordia as indicated by micro-dissection methods.

BALANCED RADIANT ENERGIES

COMPARISON OF UNFILTERED SOURCES.—Incandescent (1000-watt standard), mercury, and white and daylight fluorescent lamps (30-watt) were used over Nobel spinach, Biloxi soybean, and Indiana Baltimore tomato. The radiant energy was balanced for the various sources to approximately 800 footcandles directly under the lamps. The mercury and incandescent lamps were arranged in one plot so that there were varying proportions of mercury and incandescent radiant energy across the plot as indicated in the tables. A 15-hour photoperiod was used.

The tomato and soybean plants were seeded directly in the gravel and the

TABLE II

RESPONSE OF NOBEL SPINACH TO RADIANT ENERGY AT 800 FC FOR 15 HOURS FROM VARIOUS SOURCES WHEN GROWN AT 20° C. AND 25° C.

LAMP SOURCE	20° C.				25° C.		
	NO. PLANTS	AV. FRESH WT.	AV. DRY WT.	AV. NO. MATURED LEAVES	NO. PLANTS	AV. FRESH WT.	AV. DRY WT.
		gm.	*gm.*			*gm.*	*gm.*
Incandescent-mercury							
1*	26	5.1	0.37	9.8	15	1.8	0.14
6†	45	3.1	0.23	8.1	24	1.8	0.12
3‡	23	1.4	0.10	6.3	30	1.7	0.10
Fluorescent, white					24	1.7	0.13
Fluorescent, daylight	29	3.0	0.17	6.9	23	1.7	0.13

* 1: 7 to 25% Hg.
† 2: 25 to 86% Hg.
‡ 3: 86 to 95% Hg.

spinach in a sand-peat mixture. The spinach was transplanted to the appropriate compartments on germination. Air temperatures were controlled at 15°, 20°, and 25° C. within the limits previously designated.

The growth results are given in tables II, III, and IV. It should be noted that the growth at the various temperatures cannot be compared directly as the experiments were neither conducted simultaneously nor for the same length of time. Only the data within one temperature range can be compared.

TABLE III

RESPONSE OF BILOXI SOYBEAN TO RADIANT ENERGY AT 800 FC FOR 15 HOURS FROM VARIOUS SOURCES WHEN GROWN AT 20° C. AND 25° C.

LAMP SOURCE	20° C.			25° C.			
	NO. PLANTS	AV. FRESH WT.	AV. DRY WT.	NO. PLANTS	AV. HT.	AV. FRESH WT.	AV. DRY WT.
		gm.	*gm.*		*cm.*	*gm.*	*gm.*
Incandescent-mercury							
1*	20	6.6	0.80	27	57	8.1	1.0
2†	42	5.1	0.66	21	64	6.0	0.7
3‡	23	4.0	0.48	25	64	5.2	0.6
Fluorescent, white				20	48	7.0	0.7
Fluorescent, daylight	55	4.7	0.51	21	39	6.3	0.8

* 1: 7 to 25% Hg.
† 2: 25 to 86% Hg.
‡ 3: 86 to 95% Hg.

TABLE IV

RESPONSE OF INDIANA BALTIMORE TOMATO TO RADIANT ENERGY AT 800 FC FOR 15 HOURS FROM VARIOUS SOURCES WHEN GROWN AT DIFFERENT TEMPERATU RES

LAMP SOURCE	15° C.§				20° C.§				25° C.§			
	NO. PLANTS	AV. HT.	AV. FRESH WT.	AV. DRY WT.	NO. PLANTS	AV. HT.	AV. FRESH WT.	AV. DRY WT.	NO. PLANTS	AV. HT.	AV. FRESH WT.	AV. DRY WT.
		cm.	gm.	gm.		cm.	gm.	gm.		cm.	gm.	gm.
Incandescent-mercury												
1*	28	36.0	21.8	1.33	18	29	6.9	0.39	30	27	5.0	0.28
2†	20	35.0	16.9	0.93	28	30	7.2	0.37	26	23	3.8	0.18
3‡	33	22.0	10.5	0.57	26	21	4.8	0.23	32	18	2.3	0.12
Fluorescent, white	19	9.3	5.8	0.37	41	8	3.2	0.19	22	10	1.8	0.11
Fluorescent, daylight	26	8.3	5.7	0.35					22	9	2.1	0.11

* 1: 7 to 25% Hg.
† 2: 25 to 86% Hg.
‡ 3: 86 to 95% Hg.
§ The plants in the different temperature treatments were not of the same age when harvested, and therefore cannot be directly compared with one another.

When mixtures of incandescent and mercury arc radiant energy were given the plants, there was a decrease in height and weight as the amount of mercury arc irradiation was increased   A desirable vigorous stocky growth, retaining the increased weight feature secured under the incandescent alone, was not obtained at any mixture of the two types of irradiation

Fig 1   Nobel spinach (above) and Biloxi soybean (below) at 20° C under fluo rescent and combinations of incandescent and mercury radiation   Plant second from right had 7–25% mercury and the plant on the right, 86–95% mercury   (Tables II and III)

The greatest production of dry matter for all of the species occurred under the incandescent–low mercury combination   Here the growth was tall and spindling at 20° and 25° C   At 15° C , the plants were stocky and vigorous.

Very satisfactory growth was secured with the 800 footcandles of white and daylight fluorescent radiant energy with all of the species, except at 15°. The tomato plants were small, dark green and grew slowly at this temperature   The white fluorescent in some cases gave somewhat better growth as evidenced by increased weight and height than did the daylight, but the differences were very slight.   At 15° and 20°, the tomato plants under the

fluorescent were short and stocky, with marked anthocyanin pigmentation, a blue-green color, and long petioles and short internodes. At 25°, the color was lighter green, there was little anthocyanin, which disappeared as the plants became older, and the internodes were longer than at 15° and 20°.

TEMPERATURE MEASUREMENTS

Because of the wide variations in growth and the differences in total radiated energy, measurement of the temperature of the root media and at the under side of the leaves of tomato were made when equal total visible energy was supplied from incandescent (500 fc) and white fluorescent sources (900 fc). The temperatures at the leaf were measured with a pair of constantin-copper thermocouples and a galvanometer. The thermocouples consisted of No. 40 AWG wire. The junctions were soldered to thin vanes of silver, the cold or air junctions having a vane about 1 cm. square, and the hot or leaf junction a vane about 0.5 cm. square. The leaf junction was fastened to the underside of the leaf with cellophane adhesive tape. The air junction hung from 2 to 5 cm. below the leaf junction in the shade of the leaf. The system was calibrated by suspending the junctions in water at known differences of temperature.

The temperature at the underside of the leaves varied from 4° to 5° C. above air temperature under the fluorescent lamps. The incandescent radiant energy usually raised the temperature at the underside of the leaf 9° to 11° C. higher than air temperature. These differences occurred when the refrigeration was on in the growth chambers. Without refrigeration, the differences between air and leaf temperatures were not so great.

It is realized that this was not a measurement of the actual temperature within the leaf or at the upper surface of the leaf, and that the data secured are only indicative of the range of temperature differences occurring between the two lamp sources. Previous efforts had been made to secure leaf temperatures by inserting the wire of a thermocouple into the leaf, but the injury was considerable and it was not felt that this was an accurate measurement of the temperature occurring in an intact leaf. However, the trend and magnitude of differences were similar to those secured by the method reported above.

The temperature of the root media at 5 cm. below the surface was from 3° to 6° C. higher directly under the incandescent lamps than under the fluorescent, the variation depending on the number of hours the lamps had been on previous to measurement and factors such as the length of time following irrigation.

FILTERED MERCURY ARC AND INCANDESCENT

RADIANT ENERGY.—In view of the results secured with the mercury arc radiation showing the low efficiency of dry weight production, it was considered that possibly the strong near ultraviolet radiant energy at 3654 A and the high proportion of energy in the blue might be producing an in-

hibiting effect. In order to test this hypothesis, filters were prepared as dyed gelatin films poured on glass, which completely absorbed the 3654 A line in one case and the 3654 A line and the group of lines in the blue in the other.

The filters were prepared by mixing equal parts of dye and a gelatin stock solution, pouring the mixture on carefully levelled panes of glass, 64 cm. square, at the rate of 20 ml. of mixture (10 ml. stock gelatin and 10 ml. of dye solution) per 100 sq. cm. of glass surface. The stock gelatin solution

TABLE V

TRANSMISSION OF GELATIN FILTERS USED TO ABSORB SHORTER WAVELENGTHS OF THE MERCURY ARC SPECTRUM

WAVELENGTH	FILTER No. 1 (ABSORBING 3654 A LINE) TRANSMISSION	FILTER No. 2 (ABSORBING 3654, 4047 AND 4358 A LINES) TRANSMISSION
mμ	%	%
320	0	0
330	0	0
340	0	0
350	0	0
360	0	0
365	0	0
370	0.5	0
375	6.0	0
380	16.0	0
385	30.0	0
390	36.0	0
400	64.0	0
410	76.0	0
420	81.0	0
430		0
440		0.5
450	81.0	3.0
455		7.0
460		14.0
470		34.0
480		53.0
490		68.0
500	88.0	77.0
520		86.0

consisted of 150 gm. gelatin and 50 gm. sorbitol dissolved per liter of water. Filter No. 1, absorbing the 3564 A line, contained 25 mg. naphthol disulfonic acid and 25 mg. hydroxy naphthoic acid per 100 sq. cm. of glass surface. Filter No. 2, absorbing the 3654, 4047 and 4358 A lines, contained 25 mg. picrolonic acid per 100 sq. cm. glass surface. The transmission of the two filters are given in table V.

Four experimental conditions were set up at equal total energies, including (1) high pressure mercury arc with a film of undyed gelatin on glass interposed; (2) high pressure mercury arc filtered so that the 3654 A mercury line was absorbed; (3) high pressure mercury arc filtered so that the 3654, 4047 and 4358 A lines were absorbed; and (4) incandescent irradiance

TABLE VI

RESPONSE OF INDIANA BALTIMORE TOMATO TO THE REMOVAL OF THE SHORTER WAVELENGTHS
OF THE MERCURY ARC SPECTRUM AND TO INCANDESCENT RADIANT ENERGY

TREATMENT*	AV. FRESH WT.	AV. DRY WT.	DRY MATTER	TOP-ROOT RATIO	AV. HT.
	gm.	gm.	%		cm.
Mercury arc, unfiltered	8.7	0.43	4.9	21	24
Mercury arc, 3654 A line removed	9 3	0.46	4 9	28	26
Mercury arc, 3654, 4047, and 4358 A lines removed	1 3	0 07	5 1	11	13
Incandescent	29 3	2 42	8 2	39	77

* Twenty five plants per treatment.

of equal value in total visible radiant energy to (1). The irradiance was adjusted to an average of 270 fc for the mercury treatments and to 175 fc

FIG. 2. Response of tomato to the removal of the shorter wavelengths of the mercury arc spectrum. (1) Full radiation from 400-watt H–1 mercury arc lamp. (2) Radiation from 400-watt H–1 mercury arc lamp, 3654 A line absorbed by filter. (3) Radiation from 400-watt H–1 mercury arc lamp, 3654, 4047 and 4358 A lines absorbed by filter. (4) Radiation of equal total visible energy from an incandescent lamp.

for the incandescent.   A 15-hour photoperiod was, employed.   The mercury lamps were turned on 15 minutes prior to the incandescent so that they were at full irradiance at the beginning of the photoperiod.   The temperature was maintained at 20° C.   The treatments included 25 plants each, which were under the radiation variables from germination to harvest at the end of six weeks irradiation.

The results are given in table VI and figure 2.   These data indicate that removal of the 3654 A mercury line had no significant effect on the growth of tomato.   However, when the blue lines were removed, a very great reduction in growth and dry weight occurred.   This is to be expected since the

TABLE VII

GROWTH AND FLOWERING RESPONSE OF NOBEL SPINACH TO VARIOUS SOURCES
OF ARTIFICIAL RADIATION AT 20° C.

RADIATION TREATMENT	No. PLANTS	PLANTS FLOWER ING	AV. HT.	AV. FRESH WEIGHT		AV. DRY WEIGHT		DRY MATTER
				Tops	Roots	Tops	Roots	
SERIES I. EQUAL FOOTCANDLES RADIATION TREATMENTS BEGUN 12 DAYS FROM SEEDING FOR 55 DAYS; 18-HR. DAY								
		%	cm.	gm.	gm.	gm.	gm.	%
Incandescent 750 fc	56	5	3.0	64.5	3.2	4.2	0.28	6.6
Incandescent, water filter 750 fc	46	80	9.0	40.8	2.3	3.0	0.19	7.4
Fluorescent* 750 fc	37	100	24.4	44.3	1.3	2.5	0.09	5.6
SERIES II. EQUAL TOTAL RADIANT ENERGIES IN THE VISIBLE RADIATION TREATMENTS BEGUN 12 DAYS FROM SEEDING FOR 42 DAYS; 24-HR. DAY								
Incandescent 500 fc	45	89	26.8	28.1	1.2	2.3	0.10	8.0
Incandescent, water filter 500 fc	47	100	68.0	32.5	1.1	2.3	0.11	7.1
Fluorescent* 900 fc	48	100	31.3	24.8	0.8	1.9	0.07	7.6

* White.

blue lines of the mercury coincide with the blue maximum of the chlorophyll absorption curve.   The yellow and green lines are in the region of minimum chlorophyll absorption, and therefore, even though there is a great deal of energy radiated in these lines, it is relatively ineffective in promoting carbon assimilation and the poor growth that resulted was undoubtedly due to this low photosynthetic rate.   Under this condition, 25 per cent of the plants died before the 6 weeks of treatment were finished.

Comparison of water-filtered and unfiltered incandescent radiant energy with fluorescent radiant energy

It was thought that perhaps the tall weak growth secured with plants grown under unfiltered incandescent irradiation as compared with fluores-

cent might be partly due to the rise in temperature of the root media and to the differential increase in temperature of the leaves under the incandescent. It could be determined whether this were the case if the incandescent lamp were filtered with water so that the far infrared radiant energy were removed and no great differential increase in temperature of the leaves and the root media occurred.

TABLE VIII

GROWTH RESPONSES OF BILOXI SOYBEAN TO VARIOUS SOURCES OF ARTIFICIAL RADIATION AT 20° C.

RADIATION TREATMENT	No. PLANTS	AV. HT.	AV. FRESH WEIGHT		AV. DRY WEIGHT		DRY MATTER
			TOPS	ROOTS	TOPS	ROOTS	
SERIES I. EQUAL FOOTCANDLES HARVESTED 24 DAYS AFTER SEEDING; 18-HR. DAY							
		cm.	gm.	gm.	gm.	gm.	%
Incandescent 750 fc	55	36.4	10.0	1.32	1.36	0.12	12.2
Incandescent, water filter 750 fc	48	54.1	6.8	1.23	0.85	0.08	11.5
Fluorescent† 750 fc	51	30.5	4.7	0.96	0.54	0.06	10.6
SERIES II. EQUAL TOTAL RADIANT ENERGIES IN THE VISIBLE HARVESTED 31 DAYS AFTER SEEDING; 18-HR. DAY							
Incandescent 500 fc	42	51.2	11.6	1.10	1.70	0.13	14.4
Incandescent, water filter 500 fc	40	74.2	8.4	0.80	1.06	0.10	12.7
Fluorescent† 900 fc	40	40.3	5.0	0.58	0.62	0.05	11.8
Greenhouse*	80	34.1	6.4	1.06	0.84	0.83	12.3
SERIES III. EQUAL TOTAL RADIANT ENERGIES IN THE VISIBLE HARVESTED 34 DAYS AFTER SEEDING; 24-HR. DAY							
Incandescent 500 fc	27	77.0	30.8	1.9	4.4	0.21	14.1
Incandescent, water filter 500 fc	32	87.4	18.5	1.6	2.6	0.12	13.5
Fluorescent† 900 fc	36	66.4	14.2	1.9	2.0	0.19	13.5

* Average 10-hour winter day.
† White.

Indiana Baltimore tomato, Nobel spinach and Biloxi soybean were grown under controlled temperature and incandescent (750-watt standard) and fluorescent radiation (30-watt white) conditions as previously described and indicated in the tables. For the water-filtered incandescent, the radiant energy was filtered through five inches of water. The results for the three species are given in tables VII, VIII, and IX.

TABLE IX

GROWTH RESPONSE OF INDIANA BALTIMORE TOMATO SEEDLINGS TO EQUAL TOTAL VISIBLE
RADIANT ENERGIES FROM VARIOUS SOURCES OF ARTIFICIAL RADIATION AT 20° C.

RADIATION TREATMENT*	No. PLANTS	AV. HT.	AV. FRESH WEIGHT		AV. DRY WEIGHT		DRY MATTER
			TOPS	ROOTS	TOPS	ROOTS	
		gm.	*gm.*	*gm.*	*gm.*	*gm.*	%
Incandescent 500 fc	20	43.0	21.3	1.6	1.40	0.134	6.3
Incandescent, water filter 500 fc	20	30.6	18.3	2.3	1.18	0.153	6.0
Fluorescent† 900 fc	20	10.0	9.9	1.6	0.84	0.140	8.5

* Radiation treatments begun 10 days from seeding for 30 days; 15 hr. photoperiod.
† White.

The height and weight of the spinach plants were dependent largely on whether the flowering condition prevailed or not. When equivalent foot-candles were used from the various sources at 750 fc for 18 hours daily, only 5% of the plants under the incandescent flowered, while 80% flowered under the water-filtered incandescent and 100% under the fluorescent. The flowering results with spinach under unfiltered incandescent may have been
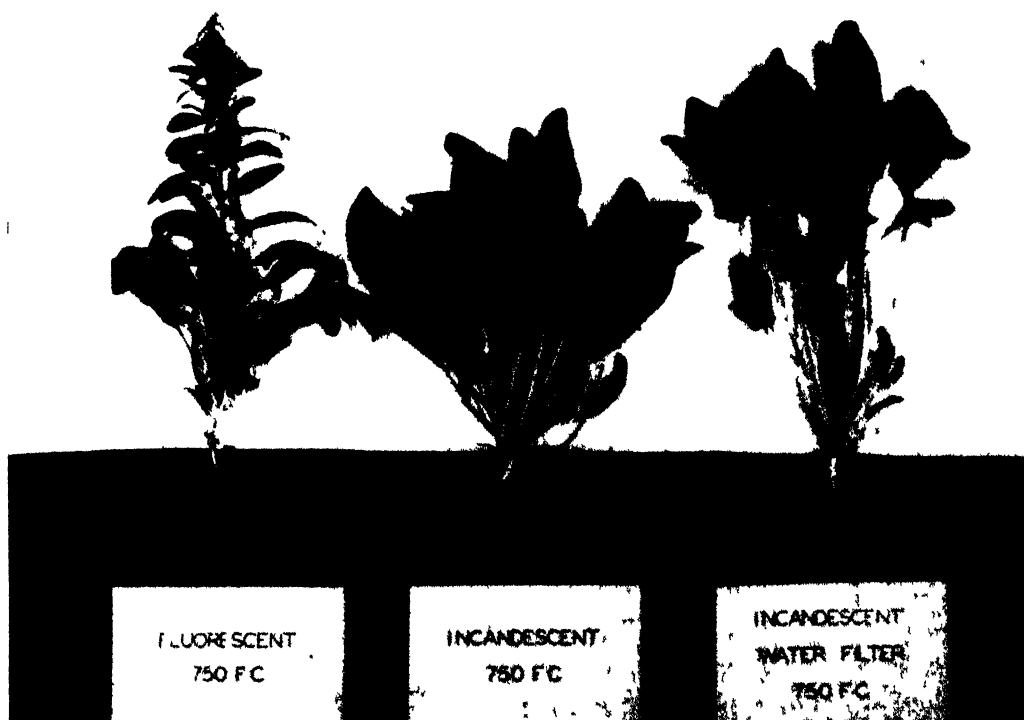
FIG. 3.   Nobel spinach at equal footcandles of white fluorescent, incandescent and water-filtered incandescent radiant energy at 20° C.   (Table VII.)

due to the comparative temperature increase, although Nobel spinach has not been shown to be temperature sensitive in its qualitative flowering responses under greenhouse conditions (21). When equal total energies in the visible were used and the plants were given 24 hours at 500 fc incandescent (filtered and unfiltered), 89% of the spinach plants flowered in the unfiltered incandescent, and 100% under the filtered incandescent and the comparable fluorescent treatment (900 fc). Where fairly comparable flowering percentages

FIG. 4. Biloxi soybean at equal footcandles of white fluorescent, incandescent and water-filtered incandescent radiant energy at 20° C. (Table VIII.)

existed, the greatest height and fresh weight of the plants occurred when the incandescent was filtered with water. The dry weights were similar for the two incandescent treatments. As was found previously, the plants under the fluorescent were shorter and had less fresh and dry weight.

Biloxi soybean gave uniform trends in growth with the various sources regardless of irradiance or daylength. The total fresh and dry weights were, in order of decreasing magnitude: incandescent, filtered incandescent,

and fluorescent.   The heights decreased from the filtered incandescent, through the incandescent to the fluorescent.   Percentage dry matter was greater for the incandescent.

Tomato was decreased in height from the filtered incandescent as compared with the incandescent, with the shortest plants in the fluorescent.   The fresh and dry weights of the tops decreased in the same order.   The heavier roots were in the fluorescent and the percentage dry matter was least with the filtered incandescent.

Fig. 5.   Indiana Baltimore tomato at equal total visible radiant energies from white fluorescent, incandescent and water-filtered incandescent sources at 20° C.   (Table IX.)

Discussion of results

The results show that, of the three sources used in this investigation, the incandescent and fluorescent lamps are the only ones which produce reasonably satisfactory growth.   The mercury arc is inefficient in supporting growth, probably due to the fact that the two most powerful lines in the visible spectrum occur in the green and yellow at 5461 and 5770–5791 A, respectively, where the chlorophylls have a minimum absorption.   The blue lines at 4047 and 4358 A are responsible for only slightly more than one-third of the total visible radiant energy of the H–1 mercury arc and are the only lines which coincide with the chlorophyll absorption bands.   These considerations appear to account in part for the low efficiency of the mercury arc.   Removal of the strong 3654 A line in the near ultraviolet did not produce any significant effect, thus indicating that this line cannot be considered as producing any appreciable depressing effect on growth.

In general, the greatest dry weight production and height occurred with incandescent sources, whether compared on the basis of equal total electrical power consumption per plant, equivalent footcandles, or equal total visible energies..   This indicates that the incandescent lamp is more efficient in the production of dry matter than either the daylight or white fluorescent lamps.

The principal disadvantage of the incandescent lamp is that it produces a plant with relatively long internodes, long petioles, and weak stems as compared with the growth obtained under solar irradiation. Data presented by POPP (**15**) and others, show that for high irradiances, blue radiant energy produces a shorter plant than red; under the longer wavelengths, the plant is tall, has long internodes, and is somewhat etiolated in appearance. However, supplementing the incandescent lamp with mercury arc irradiation did not appreciably improve the situation. Possibly supplementing the incandescent lamp with a blue-rich source as the blue fluorescent might prove useful in this connection, but this was not tried in these experiments.

A second disadvantage of the incandescent is that the radiated infrared causes the temperature of the leaves and root media to increase appreciably above the air temperature. There are several alternatives which can be used to partially correct this situation. The radiant energy can be filtered through water, which removes a little over one-third of the total radiated energy. Also the growth chambers can be operated at a reduced temperature to partially compensate for the higher leaf and media temperatures. Adequate circulation of air is necessary with high wattage unfiltered incandescent lamps. Where constant temperature conditions cannot be maintained, it is practically impossible to use unfiltered incandescent lamps to furnish the sole radiant energy for plants growing in small enclosed areas.

The temperature effects do not appear to be primarily responsible for the tall weak plants, elongated internodes, and long petioles secured with the incandescent. Removing the far infrared from the incandescent source with water so that the temperatures of the leaves and root media were not appreciably increased, as compared with fluorescent irradiation, appeared, in some cases, to increase the magnitude of the etiolated response rather than to decrease it. However, the temperature differences are responsible, in all probability, for some of the differences in synthesis of dry matter and for certain of the growth responses.

The plants were shorter, stockier and had darker green leaves with the fluorescent sources used than with the incandescent, and a superior growth form despite the fact that the total dry matter produced was considerably less than that produced under the incandescent. The fluorescent lamps have a somewhat larger proportion of the total energy emitted in the visible where it is useful to the plant than does the incandescent. A lesser proportion is in the infrared and much of this is dissipated by convection and conduction so that the lamps may be placed much closer to the plants than is possible with incandescent sources. With 30-watt fluorescent lamps, 3500° K. white, it is possible to secure 1200 footcandles directly beneath the lamps, if the lamps are mounted on a white reflecting surface at 1½-inch centers. A choice of fluorescent lamps is available so that a differential distribution of wavelengths is possible. However, none of the lamps can be used for critical spectral studies since their transmission bands are not sharp enough for most work of this type.

On the whole, an entirely satisfactory artificial source of radiant energy for plant growth is not yet available. However, the fluorescent sources appear to best meet requirements for general experimental work where radiant energy conditions are not to be a variable, despite the slower growth rates secured, since the plant form more nearly resembles that secured with daylight. Combinations of fluorescent lamps employing various phosphors may give improved results as compared to either the white or daylight, especially if a transmission curve similar to the chlorophyll absorption curve or the photosynthesis curve could be secured.

It should be pointed out as a consideration in working with artificial sources, that experimental data secured with any artificial source cannot be directly applied to interpretation of phenomena observed with solar radiant energy. Because no source has the same spectral distribution of energy as sunlight, it is impossible to predict that plant responses to such variables as photoperiod, irradiance, and others will be similar to those secured under greenhouse or field conditions.

Summary

Comparative growth responses of certain herbaceous annuals to several sources of artificial radiant energy were investigated. The sources included unfiltered incandescent, water filtered incandescent, high pressure mercury arc lamps, and white and daylight fluorescent lamps. The plant material irradiated was: China aster, variety Heart of France; spinach, variety Nobel; soybean, variety Biloxi; and tomato, variety Indiana Baltimore. The studies were made at 15°, 20°, and 25° C. and at varying photoperiods.

The greatest height and fresh and dry weight occurred with the incandescent sources. The water-filtered incandescent condition often produced taller plants with a lower percentage of dry weight than was the case with the unfiltered incandescent. The shortest plants, with the smallest fresh and dry weights, occurred with the high pressure mercury arc lamp. Removing the 3654 A line in the near ultraviolet spectrum of the mercury arc lamp failed to produce any significant increase in growth, indicating that this line is not responsible for the poor growth secured with this source. When the incandescent and mercury sources were set up in a single plot so that there were varying percentages of mercury and incandescent radiation, the height and weight decreased as the percentage of mercury radiant energy was increased. The plants under the fluorescent lamps were of an intermediate type of growth which was vigorous and stocky except at 15°. At 15°, the tomato plants were hard and grew slowly, a typical low temperature response. The white fluorescent gave a very slightly increased growth in some cases as compared with the daylight fluorescent lamp.

While there was greater production in fresh and dry weight under the incandescent lamp sources, the growth, on a whole, was tall and spindling except at 15° C. with tomato, and was not of a desirable type for general studies. Filtering the incandescent lamps through water to eliminate the

differential rise in temperature of the leaves and root media as compared with the fluorescent lamps failed to produce a stockier growth, but rather accentuated the etiolated type of response in some cases. It appears that the differences in growth responses secured with the various sources can be correlated principally with the differences in the respective spectral characteristics of the lamps.

The same trend in the results as summarized above were secured whether the energies were balanced on the basis of equal power consumption per square foot, equivalent footcandles, or equal total visible energies. The general trend was the same for 15-, 18-, and 24-hour photoperiods for the plant material used, except where differential flowering responses were secured.

It is concluded that no entirely satisfactory artificial sources of radiant energy for plant growth are available, but that the fluorescent sources appear to best meet requirements for general experimental work.

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THE THERMODYNAMICS OF ACTIVE (NON–OSMOTIC) WATER ABSORPTION

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In recent years, several workers (1, 2, 12, 14, 15) have brought forward evidence of a "secretion pressure" or "non-osmotic force" which is supposed to control the movement of water into (and out of) living plant tissues by causing an "active" (non-osmotic) uptake of water. So much of this evidence is indirect and controversial that papers expressing the opposite views have appeared (6, 7, 17). Since the postulated phenomenon depends on the expenditure of respiratory energy, it seems advisable to determine whether the conclusions are thermodynamically possible.

Perhaps the strongest argument in favor of active water absorption is the recognized fact of active solute absorption. It seems axiomatic that if a plant is capable of the latter, it should also be capable of the former. The concentration of K ions in the cell sap of Valonia has been shown to be 20 parts per thousand in excess of that outside the cell (20). If we assume a similar excess of a corresponding anion, this may mean a diffusion pressure of as high as 15–20 atms. However, Valonia cells have a volume of one to several cm.^{3} If we assume that the cells of most higher plants are spherical with a radius of 5–50 μ (or non-spherical and of larger dimensions) the specific area $\left(\dfrac{\text{area}}{\text{volume}} \text{ or } \dfrac{1}{\text{radius}} \right)$ is of the order of 200 to 2000 times that of Valonia cells. Consequently, the cells of higher plants would have to expend 200 to 2000 times as much energy per unit volume as Valonia cells in order to maintain such a gradient.

The values obtained for higher plants are somewhat less | 100 milliequivalents K per liter in excess of that in the surrounding solution (9) |; this is equivalent to a pressure of about 4½ atms. This gradient, however, is between the root cells and the surrounding soil solution. Consequently, the area separating the diffusion gradient is immensely smaller than the total cell area of a plant. In order to maintain a similar gradient between the protoplasm and vacuole of plant cells, it would require the expenditure of thousands of times as much energy as to maintain the above K gradient between roots and soil.

There is another factor, however, that must be considered. The energy required to maintain a negative gradient for any substance is proportional to the permeability of the cell to that substance; i.e., to the rate of diffusion of the substance across the membrane separating the two different concentrations. Since the cell is far more permeable to water than to any other substance normally occurring in it, far more energy would have to be expended to maintain a negative gradient for water than for any other substance.

The permeability constant for water is of the order of 10^{-7} M/cm.^{2}/sec. for a gradient of 1 M; that for K is of the order of 10^{-12} to 10^{-14} M/cm.^{2}/sec. for gradients of 0.01 to 0.06 M [(4) tables XIV and XXXI]. Therefore, at least 1000 times as much energy would have to be expended to maintain the same negative gradient for water as for potassium, which is one of the most rapidly penetrating ions.

Thus the energy used to maintain the high concentration of potassium in plant cells is sufficient to maintain a diffusion gradient for water of only the most minute fraction of one atmosphere.

If a negative diffusion gradient is to be maintained, some mechanism must be available to "pump" water against the gradient as rapidly as it is diffusing with the gradient. Consequently, work must be done equal to the product of the force × the distance moved:

$$W = Fs \tag{1}$$

Since by definition $F = pa$ and $s = vt$

$$W = pavt \tag{2}$$

where p = the negative diffusion pressure against which water is moved; i.e., the "active" pressure.

a = the area across which the gradient occurs.

v = velocity of diffusion of water.

t = time.

In order to maintain cgs units, p (the pressure in atms.) must be converted to dynes/cm.^{2}; i.e., × 10^6. If A is taken as specific area, the resulting quantity is the work done/cm.^{3} tissue or /gram tissue (approximately); v, the velocity of diffusion of water, can be obtained from the permeability of the cell to water. This value has been determined for several plant cells. When expressed in μ hr. atm. at 20° C., it is 10–20 for Fucus eggs (16); 33 for Salvinia (10); and 21 for onion bulb cells (11). Since these values do not differ greatly from each other, a value of 20 will be used. Converting to cm., it becomes 20×10^{4} cm. /hr. atm. From this value, the velocity of diffusion v, becomes Pp where P = permeability and p = diffusion gradient in atms. Thus

$$W = 10^6 paPp = 10^6 p^2 AP \text{ ergs/hr., gm. tissue} \tag{3}$$

The only source of energy in the plant for such work is the respiratory process. Consequently, in order to determine whether any particular negative gradient is thermodynamically possible, it is necessary to calculate the amount of sugar that would have to be respired in order to do this work.

Taking 4.185 as the mechanical equivalent of heat and 3.75 kg. cal./gm. as the heat of combustion of glucose, the amount of sugar respired would be

$$S = \frac{10^6 p^2 AP}{10^7 \times 4.185 \times 3.75 \times 10^3} = 6.4 \times 10^{-6} p^2 AP \tag{4}$$

or, if P is taken as 2×10^{-3} cm./hr./atm.

$$S = 1.28 \times 10^{-8} p^2 A \tag{5}$$

This gives the gm. glucose/gm. tissue/hr. that would have to be respired in order to maintain the negative diffusion gradient of p atms.

It should be noted that this is a minimum value, assuming that all the respiratory energy released is utilized for this purpose; i.e., 100% efficiency. And it does not matter what the mechanism of active absorption is, this minimum amount of energy must be utilized.

Analysis of published results

BENNET-CLARK et al. (1) compared the plasmolytic and cryoscopic values for the osmotic pressures of six kinds of plants. In most cases the plasmolytic values were about 50% higher than the cryoscopic values, though some plants yielded no significant differences. They concluded that the difference is due to a "secretion pressure." According to their calculations, the "secretion pressure" amounted to 2.6–7.1 atms. in the case of the beets they tested. Thus p can be taken as having an average value of 5 atms. A, the specific area, is the tonoplast area/cm.^{3} Beet root cells are large, but the largest are not more than 0.5 mm. long and 0.2 mm. wide. They are not perfect cylinders, but if we use these maximum figures and assume them to be such, we shall certainly be underestimating the specific area. The cytoplasm layer is barely detectable in these cells, consequently cell area can be used for tonoplast area. From these values, specific area of beet cells (A) can be estimated.

$$A = \frac{2\pi rl + 2\pi r^2}{\pi r^2 l} = \frac{2(1+r)}{rl}.$$

where l = .05 cm.

r = .01 cm.

A = 240 cm.^{2}/cm.^{3}

If we now apply equation (5)

S = 7.7 × 10^{-5} gm. glucose/gm. tissue/hr.

Since the dry matter of a beet is about 15% of the fresh weight, this means that the beet would respire all of its dry matter in less than 3 months in order to maintain the negative diffusion gradient of 5 atms., even if all the respiratory energy were used for this one purpose.

In a later paper, BENNET-CLARK and BEXON (3) determine the respiratory rate of beet tissue. The highest values obtained by them were about 150 μl gas/hr./gm. fresh tissue. In terms of glucose utilized, this is equivalent to 2 × 10^{-4} gm. glucose/gm. tissue/hr. Consequently, even the maximum respiration rate of beet tissue, which occurs temporarily under conditions of stimulation, is only about 2.5 times the rate that would be required to maintain the negative diffusion gradient postulated by BENNET-CLARK et al., if all the respiratory energy released were utilized for this sole purpose.

· These calculations prove that their hypothesis of a "secretion pressure" cannot account for the differences they obtained between plasmolytic and cryoscopic values. The true explanation is therefore to be sought elsewhere.

Other workers, following up their results, have also found differences between the plasmolytic and cryoscopic values. 'ROBERTS and STYLES (17) obtained good agreement in the case of some conifer leaves but large differences with others. They attribute the differences to the presence of mucilaginous colloid. CURRIER (6) repeated the work with beets. In most cases the plasmolytic values were no more than 10% larger than the cryoscopic values, though in one series he obtained the same 50% difference found by BENNET-CLARK et al. His explanation is similar to that of ROBERTS and STYLES. He suggests that water squeezed out of the wall and protoplasm dilute the sap expressed from the frozen and thawed tissues. His own calculations, however, show that the protoplasm and wall do not contain enough water to account for the difference. Similarly, the colloid in the plants used by ROBERTS and STYLES cannot hold enough water to account for the differences of as much as 21.5 atms. that they obtained.

To check the experimental results of BENNET-CLARK et al., the author determined the plasmolytic and cryoscopic values for red beets. A whole

TABLE I

CRYOSCOPIC AND PLASMOLYTIC O.P. VALUES FOR BEETS

BEETROOT	Δ JUICE	Δ ISOTONIC CaCl$_2$	$\dfrac{\Delta \text{ ISOTONIC CaCl}_2}{\Delta \text{ JUICE}}$
	°C.	°C.	
1	0.885	1.02	
2	0.82	1.02	
3	0.91	0.99	
4	0.885	1.02	
5	0.93	0.99	
6	0.89	1.02	
Total	5.32	6.06	1.14

beet was sliced, sections were cut from near the middle of the center slice for plasmolytic determinations, and the other slices were frozen for cryoscopic determinations.

In agreement with BENNET-CLARK et al., the plasmolytic method yielded higher values than the cryoscopic (table I). But the increase is only 14% instead of the 50% obtained by them. Similar results were obtained with a set of beets having a freezing point lowering of about 1.5° C. It has long been known that such a difference exists, but this has usually been ascribed to the fact that the cryoscopic value is obtained with the normal cell sap, the plasmolytic value with sap concentrated by the exosmosis of water accompanying the loss of turgor on plasmolysis.

BENNET-CLARK et al. state that the ratio of the volumes of turgid : flaccid cell can be obtained from measurements of the linear dimensions of thin slices before and after plasmolysis. According to them, "in the case of the three beets used, the shrinkage was found to be between 2 and 4%." From this they conclude that the value of the ratio in the beet roots examined was between 1.00 and 1.05.

It is not clear from their statement whether the volume shrinkage or linear shrinkage is between 2 and 4%. If it is the latter, then the volume ratio will be between 1.06 and 1.12 instead of 1.00 and 1.05. This would agree better with results usually obtained for potato tuber tissue. Thus the two ratios calculated from LYON's (12) results (table I) are 1.04 and 1.13, respectively.

To check this, some measurements were made on beet roots (table II) taken from the same lot as those used for the determinations in table I.

The average linear ratio was 1.05, which agrees with the values given by BENNET-CLARK et al. for the volume ratio. The true value for the volume ratio is 1.16. Thus the plasmolytic value is 14% larger than the cryoscopic value, and the decrease in cell volume that occurs during the determination of the plasmolytic value is 16%. Consequently, in these beets the "secretion pressure" postulated by BENNET-CLARK et al. is non-existent.

From these results it is evident that part of the discrepancy between the plasmolytic and cryoscopic values of BENNET-CLARK et al. is due to their

TABLE II

DETERMINATIONS OF THE RATIO OF TURGID : FLACCID CELL IN BEETROOTS.
EACH VALUE AN AVERAGE OF 6–10 STRIPS

ORIGINAL LENGTH OF TISSUE STRIP	LENGTH IN HYPERTONIC CaCl$_2$	LINEAR RATIO	VOLUME RATIO
mm.			
30.0	28.50	1.055	
29.0	27.75	1.045	
29.5	28.15	1.050	Av. 1.16

underestimation of the change in cell volume on loss of turgidity. CURRIER's values (2.2–7.3%) also seem too low, possibly due to the fact that one measurement was made on normal tissue, the other on infiltrated tissue. Changes in intercellular volume may conceivably mask part of the change in cell volume. Judging from cell measurements, however, the maximum cell shrinkage is not likely to be more than 20%, and therefore cannot wholly account for the differences obtained by BENNET-CLARK et al.

The method they used for plasmolytic determinations may also have been at fault. They deny the possibility of adhesion of protoplast to cell wall, partly because no wall deformation was seen. Such a deformation is not likely to be seen since it would be in the vertical plane of the section. The method used by the author avoids such an error, since the cells are always strongly plasmolyzed first, then allowed to deplasmolyze in successively weaker solutions until incipient plasmolysis is detected. This method has the added advantage of producing convex plasmolysis even in cells with the most viscous protoplasm. Traces of convex plasmolysis are much more readily detected than traces of concave plasmolysis. The use of CaCl$_2$ as plasmolyte avoids changes in concentration such as occur in sugar solutions due to the activity of microorganisms. The more rapid diffusion of CaCl$_2$ permits equilibrium to be reached much sooner than with sugar solutions.

BENNET-CLARK *et al.* do not mention whether or not they determined the freezing point lowerings of the sugar solutions used. If the osmotic pressures were simply calculated from the concentration, the plasmolytic values are almost certain to be high.

The wide spread in their plasmolytic values for different cells of the same root may indicate some factor such as adhesion or simply too great a variability between cells to obtain an average for the whole tissue from the 100 cells examined. The author always found it possible to distinguish three solutions: a hypertonic solution producing slight plasmolysis in all the cells; an isotonic solution producing incipient plasmolysis in about half the cells; and a hypotonic solution producing plasmolysis in few or none of the cells. The isotonic solution usually differed from the other two by about $\frac{1}{2}$ atm.

There are several other possible explanations. The smaller samples used by them to determine the freezing point lowering of the juice may have introduced larger errors because of the condensation of moisture on the cold tissues or due to inability to express a large enough fraction of the juice from such small samples to be representative (the first fraction would be more dilute). Thus their results varied from 0.67 to 0.83° C. for six different cylinders obtained from the same beet. The variations in our results for six different beets were only half as great.

Thus there are many sources of error, the sum total of which might conceivably lead to large differences such as were obtained by BENNET-CLARK *et al.* That these differences are not real is indicated by thermodynamic calculations, by our failure to obtain them, and by the great variability in the results obtained by BENNET-CLARK *et al.*

MASON and PHILLIS (**14**) assumed that the juice expressed from living leaves was the vacuolar sap and that remaining in the leaves was protoplasmic. On the basis of this assumption, they found that the O.P. of the "vacuolar sap" of cotton leaves was 2.4 atms., that of the "protoplasm" 19.3 atms. They concluded that the difference represented a "secretion pressure."

MASON and PHILLIS used the blades of cotton leaves freed of large veins. The cells are mainly cylindrical and not more than 100μ long by 20μ in diameter. From these dimensions, the specific area (A) is 2200 cm.²/gm. Consequently from equation (5)

$$S = 8.1 \times 10^{-3} \text{ gm. glucose gm. tissue hr.}$$

From MASON and PHILLIS' own measurements of the respiration rate, it can be calculated that 0.088 gm. dextrose were respired/100 gm. tissue/hr.; i.e., the actual rate of respiration is about $\frac{1}{10}$ as rapid as would be required to maintain the negative diffusion gradient even if all the respiratory energy released were used for this sole purpose.

The fantastically high amount of energy that would have to be expended by the protoplasm in maintaining this negative diffusion gradient makes it quite clear that MASON and PHILLIS' "vacuolar" and "protoplasmic" fractions are in no way related to the true vacuolar and protoplasmic components in the living cell.

MASON and PHILLIS postulated that the pressure produced protoplasmic "fissures" which allowed the passage of unaltered vacuolar sap through the protoplasm.   They give no reason for this hypothesis other than that it fitted in with their other assumption that the sap is unaltered by passage through the living cells.   The fact that the concentration of the juice remained constant as the pressure was increased indicates that no such fissures developed, since they would enlarge with the increased pressure.   Furthermore, since it required pressures of 400 atms. (6000 lb./in.^{2}) to obtain the expressed juice, and since the living cells plasmolyzed in a solution with an O.P. of 22.8 atms., it is inconceivable that these pressures should fail to force water through the unaltered plasma membrane.   Consequently, their "vacuolar sap" was really almost pure water which they had filtered through the plasma membrane.

Using two methods of calculation (both based on the assumption that the liquid expressed from living tissues represents vacuolar sap) they conclude that the cell consists of 70% protoplasm and 30% vacuole.

CHIBNALL (5) gives analytical values for spinach leaves from which these fractions can be calculated.   The total protein N varied from 2.9–3.8% of the dry weight during a period in which the dry weight varied from 16–11% of the fresh weight.   On a fresh weight basis, this gives a value of 0.45% N.   But only $\frac{1}{3}$ of this was cytoplasmic N, the rest being chloroplast N.   And since the cytoplasmic protein had an N content of 16.25%, the amount of cytoplasmic protein was therefore 1.385 gm./100 gm. fresh tissue.   From analyses of the protoplasm of many organisms, it has been found that it contains 7–10% protein (19).   This gives a value of 19.8–13.85 gm. cytoplasm/100 gm. fresh tissue.   The cytoplasm of spinach leaves therefore cannot occupy more than $\frac{1}{5}$–$\frac{1}{7}$ of the cell volume.   The true value is likely to be even lower than this since, in view of the high O.P. of leaf cells, the cytoplasm of leaves is undoubtedly less highly hydrated than that of the slime moulds and animals on which the 7–10% value is based.

Whether or not the same values would be obtained with cotton leaves, we cannot say.   MASKELL and MASON (13) give N values of 1% of the fresh weight for cotton leaves, which is double the value used above.   However, this represents total N, which must be considerably higher than protein N.   The values for cotton leaves are therefore probably of the same order as those for spinach.

Observational methods also show quite clearly that their calculated results are in error.   They claim to have observed that the protoplasm occupies more of the cell than does the vacuole.   To check on their observations, the author vitally stained sections of cotton leaves with neutral red.   As in most leaves, practically the whole protoplast stained, the cytoplasm forming an almost invisible layer around the stained vacuole.

Every experimental value obtained by them gives further evidence of their misinterpretation.   Thus the expressed juice had a pH of 7.0 to 7.2, the residue 5.7.   Since protoplasm is known to have a pH about 7.0 (8) and

vacuolar sap is always acid [usually below pH 6 (**18**)] the above fractions cannot be vacuolar sap and protoplasm respectively.

BENNET-CLARK et al. (**2**) used MASON and PHILLIS' method to express juice from leaves containing colored vacuolar sap. The presence or absence of color in the expressed juice enabled them to judge whether or not they obtained vacuolar sap. When the pressures were applied gradually, the expressed juice was colorless. This juice was therefore almost pure water, filtered through the semi-permeable protoplasm layers. When, however, the leaves were subjected to sudden large increments of pressure, the expressed juice was strongly colored and had considerably higher osmotic values (7.86 atms. for *Fagus sylvatica* when 156 atms. pressure was suddenly applied; 3.75 atms. when 174 atms. pressure was applied gradually). Juice expressed from the killed residue (frozen in dry ice) was less intensely colored in the case of *Fagus sylvatica* and had higher osmotic values. BENNET-CLARK et al. conclude that MASON and PHILLIS' interpretation is correct when pressures are applied suddenly; i.e., the juice from live tissue represents vacuolar sap and that from the killed residue cytoplasmic sap. They also accept MASON and PHILLIS' concept of protoplasmic "fissures," through which the sap supposedly passes unaltered.

On the basis of these assumptions, they obtained differences of 6–19 atms. between the osmotic pressures of the "cytoplasmic sap" and "vacuolar sap." These results are of the same order as those of MASON and PHILLIS; consequently, the amount of energy required to maintain the negative diffusion gradient is again fantastically high.

The reasons for their interpretation are not given. It is obvious that if water can be filtered through the protoplasm when pressures are gradually applied (which BENNET-CLARK et al. proved), it will also be filtered through the protoplasm if those same pressures are suddenly applied. If the sudden application of pressure should produce protoplasmic "fissures" small enough not to injure the cells, then the expressed juice would be a mixture of unaltered or relatively little altered sap and filtered sap. The greater the pressure applied, presumably the greater the proportion passing through the fissures. Increased pressures would then result in increased osmotic pressures which is precisely what BENNET-CLARK et al. obtained. The mere fact that successive fractions *always* gave higher O.P. values is proof that they did not obtain unaltered vacuolar sap. The juice expressed from killed residue must therefore have a higher O.P. than that pressed from the live tissue. This result is in no way connected with the O.P. of the vacuole and cytoplasm respectively.

Since they never expressed all the juice (total amounts from 28 to 72% of the fresh weight were obtained) it is obvious that the average O.P. of all the expressed fractions would have to be considerably lower than that of the normal cell. Here again, this is simply due to the partial filtration of the juice and there is no need to seek any other explanation of the fact that plasmolytic values were higher than these averages.

It is interesting to note that of the six species tested by them, only one (*Fagus sylvatica*) gave higher concentrations of anthocyanin in the juice expressed from the living leaves than from the killed residue. In spite of this, BENNET-CLARK *et al.* conclude that the former represents vacuolar sap, the latter protoplasmic sap.

The above calculations prove that due to the enormous specific surface of the cell and its very high permeability to water, the living cell is unable to maintain a diffusion gradient of several atmospheres between the protoplasmic water and vacuolar water. In the case of the beet tissue used by BENNET-CLARK *et al.*, a gradient of 1 atmosphere would require fully 1/60 of the total maximum respiratory energy released under temporary stimulation or 1/30 when respiration is at the normal rate. In the case of the cotton leaves used by MASON and PHILLIS, 1/30 of the total respiratory energy released would be just sufficient to maintain this same 1 atmosphere gradient. Since so many other energy-consuming processes are simultaneously taking place in the cell, it is doubtful if this large fraction could be so used, and we can conclude that the energy available is certainly incapable of maintaining a diffusion gradient of more than 1 atm. between the cell sap and protoplasm. Since, however, the energy needed is directly proportional to the square of the gradient (equation 4) enough would be available to maintain a gradient of a fraction of an atmosphere. Whether the mechanism for this process exists in the cell is another question.

LYON (**12**) determined the "non-osmotic force" from the difference between the "calculated net osmotic pressure" and the "observed net osmotic pressure." Aside from the impossibility of obtaining a force by subtracting one pressure from another, it would be less confusing if we compared the two calculated values of wall pressure, for it is really these that he is comparing. We will avoid the use of LYON's symbols.

One value is calculated on the assumption that the wall pressure equals the osmotic pressure (O) minus the "net osmotic pressure"; i.e., the suction pressure (S) or diffusion pressure deficit. We will call this W_1.

$$W_1 = O - S \qquad\qquad (6)$$

The other is calculated on the assumption (not stated by LYON) that Hooke's law holds for the cell wall; i.e., wall pressure is proportional to wall extension (and therefore approximately to cell volume). We will call this W_2.

Let us assume with LYON that there is a "non-osmotic force" producing an "active" uptake of water. We will use the symbol A for the pressure produced by this "active" uptake. Then

$$W_1 = O + A - S \qquad\qquad (7)$$

On the basis of LYON's hypothesis, W_1 would be the incorrect value, W_2 and W_3 the correct ones. Therefore $W_2 = W_3$.

But $W_3 > W_1$ (by an amount equal to A).

Therefore $W_2 > W_1$.

Actually LYON finds that $W_1 > W_2$; e.g., in table I $W_1 = 10.35$ and

$W_2 = 5.77$. It follows that he has no evidence whatsoever for a "non-osmotic force" unless it has a negative value; i.e., the water would be pumped out of the cell instead of absorbed!

This does not, of course, explain why he did obtain such large differences between W_1 and W_2. If we consider the assumption on which W_2 is based, it can be seen that this is valid only if the wall is not stretched beyond its elastic limit, for then Hooke's law does not hold.

To determine whether or not this is true, the author tested LYON's technique. Strips of potato tissue ($20 \times 1 \times 1$ mm.) were allowed to shrink in isotonic dextrose and measured from time to time. At the end of one hour (which was found to be sufficient for equilibrium to be reached), the strips were transferred to tap water. They were measured at the end of four hours (the time used by LYON) and then returned to the isotonic dextrose. Equilibrium was again reached at the end of one hour, but to make sure, the final measurements were made after 18 hours. These checked with the ones at the end of 1 hour. The results are shown in table III.

TABLE III

LENGTH OF STRIPS OF POTATO TUBER TISSUE (AVERAGE OF 6)

ISOTONIC DEXTROSE (0.60 M)	WATER	2ND IMMERSION IN ISOTONIC DEXTROSE
mm.	mm.	mm.
20.5	24.5	22.0

Thus the elastic stretch at the end of 4 hours in tap water was only 62.5% of the total stretch. LYON's value for W_2 is 56% of the value obtained from the classical equation $W = O - S$, or 58% if he had calculated wall pressure from the increase in length of the strip instead of the increase in volume (Hooke's law refers to a linear stretch). Now if the total stretch were multiplied by 0.625 to eliminate the fraction due to plastic stretch, there would be no significant difference between the two values for W. The two percentages agree well enough, considering that they were obtained with different tubers.

It is obvious, therefore, that wall pressure cannot be calculated on the assumption that Hooke's law holds, unless a correction is made for plastic stretch (permanent set). If, however, the cell wall of the normally turgid potato tuber cell is not stretched beyond its elastic limit, a correction such as made above should be valid.

If such a correction is made, it should be possible to determine the true value for A in equation (7). But does this quantity A really represent the postulated pressure due to "active" absorption of water? It must be remembered that the quantity O represents not the osmotic value of the cell sap but of the living protoplast, since it was determined by the method of minimum cell volume. Consequently, any pressure due to active absorption of water

is included in this quantity. Any further work along the lines attempted by LYON will therefore fail to shed light on the problem of "active" water absorption.

In contrast to the above investigators, VAN OVERBEEK (15) has produced strong evidence to show that the root pressure of a growing tomato plant is partly due to non-osmotic forces. He obtained small negative diffusion gradients ($1-1\frac{1}{2}$ atms.) between the osmotic pressure of the xylem exudate and the suction pressure of the roots surrounding it. These differences were obliterated by KCN. The plants used were growing actively and therefore were in a high state of metabolic activity. The area across which the gradient was maintained was simply the area of the stele or endodermal layer. The specific surface is $\frac{2r_s}{r_r^2}$ where r_s = stele radius and r_r = root radius. Assuming the ratio $\frac{\text{stele radius}}{\text{root radius}}$ has a value of about $\frac{1}{5}$, the specific surface is therefore 0.22 cm.^{2}/cm.^{3} root tissue. This is only 1 1000-1/10,000 that of the tonoplast specific surface calculated above.

From equation (5), we can calculate that the amount of sugar that would have to be respired in order to maintain the negative gradient in VAN OVERBEEK's experiments is 2.82×10^{9} gm. gm. tissue hr.

It would therefore require the energy released by the respiration of approximately 0.3 micrograms sugar 100 gm. tissue hr. to maintain this negative diffusion gradient. Thus from the point of view of energy requirement, VAN OVERBEEK's results are well within the realm of possibility.

Summary

1. A simple formula is derived for calculating the energy needed to maintain a negative diffusion gradient in plant tissues.

2. Calculations reveal that respiratory energy is incapable of maintaining a negative gradient of more than 1 atmosphere between the osmotic pressures of the vacuole and cytoplasm of a cell.

3. A critical examination of the higher values obtained by other workers reveals that these values are due to incorrect interpretations.

4. In absorbing roots, a negative gradient of 1 atm. between the stele and the tissues external to the stele could be maintained by the energy released from the respiration of 0.3 micrograms sugar/100 gm. tissue/hr.

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EFFECT OF ALTERNATING CONDITIONS OF BORON NUTRITION UPON GROWTH AND BORON CONTENT OF GRAPE VINES IN SAND CULTURE[1]

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(WITH THREE FIGURES)

The effect of boron deficiency upon the growth and fruiting of the grape under field conditions has previously been reported by the author (**9, 10**). The field investigations led to further studies under controlled conditions; thus the present paper is concerned with the development of symptoms of boron deficiency and the accumulation and translocation of boron in the vine under alternating levels of boron nutrition of grape vines in sand culture.

Methods

Two-year-old vines of the varieties Catawba, Herbert, Golden Muscat, and Lindley were planted in 2.5-gallon glazed crocks filled with twenty-mesh washed white quartz sand and provided with a drainage hole. On March 23, 1943, twelve vines of each variety, carefully selected as to uniformity of size, were planted one to a crock. The vines were pruned back to two canes, and four buds were permitted to develop shoots on each vine. The crocks were arranged on a ground plot in the greenhouse in four varietal blocks of twelve vines each.

Two nutrient treatments, hereafter designated as "plus-boron" and "minus-boron," were used. The plus-boron solution contained: 170 p.p.m. of mono-potassium phosphate; 590 p.p.m. of calcium nitrate; 148 p.p.m. of magnesium sulfate; 5 p.p.m. of iron citrate; 0.91 p.p.m. of manganese chloride; 0.11 p.p.m. of zinc sulfate; 0.04 p.p.m. of copper sulfate; 0.045 p.p.m. of molybdic acid; and 0.5 p.p.m. of boron from boric acid. The minus-boron solution was identical in composition except for the omission of boron. The pH of the solutions was adjusted to approximately 6.0 by the addition of sodium hydroxide. The nutrient solutions were applied by flushing each crock with 500 ml. of the solution three times a week. On other days the plants were given distilled water.

The plus-boron and minus-boron treatments were applied to alternate vines in each varietal block. Seven weeks after planting, three of the plus-boron Golden Muscat vines were changed to minus-boron treatment and three minus-boron vines were changed to plus-boron treatment. A week later a similar change was made with the Catawba and Herbert vines. The nutrient-sand culture seemed well adapted for grapes since the plus-boron vines exhibited vigorous, normal foliage and each vine made a total shoot growth

of ten to fifteen feet by the end of three months which is comparable to good field growth in the first season after planting.

Estimation of boron was made by the quinalizarin method (6). Since determinations of boron content of the vines under field conditions (10) had shown rather extreme differences existing in various parts of the vine; four classes of material were sampled for analysis: (a) lower leaves, consisting of mature leaves toward the base of the cane usually including the second to fourth leaves; (b) upper leaves, consisting of the four or five young, smaller leaves at the end of the shoot; (c) lower stem, the portion subtending the lower leaves; and (d) upper stem, that portion subtending the upper leaves. The upper leaves of class (b) were in actively growing condition in all normal plus-boron vines but were much older and apparently had ceased growth in the boron-deficient vines. All leaf samples included the petiole. Material from the three vines constituting a nutrient treatment were composited to form a sample. All samples were taken at the conclusion of the experiment (three months after planting) with the exception of a series taken from the Catawba and Herbert vines at the time of changing from plus to minus and from minus to plus boron nutrition.

Results

DEFICIENCY SYMPTOMS

The first evidence of symptoms of boron deficiency were observed about one month after growth commenced. At this time the plus-boron Catawba vines had made a total shoot growth of about 120 cm., the Golden Muscat vines 70 cm., the Herbert vines 110 cm., and the Lindley vines 155 cm. The symptoms in the early stages were exhibited as (a) a diffuse yellowing or chlorosis of the younger leaves; (b) brownish, water-soaked areas developing in the apical tendrils; and (c) cupping of the third or fourth leaf from the shoot tip. The exact order of appearance of these symptoms varied among the varieties. The Golden Muscat vines first showed chlorosis while with the Catawba and Herbert vines, the water-soaked areas on the young tendrils appeared first. Later stages of the deficiency consisted largely of a progressive worsening of the earlier symptoms shown on the leaves and tendrils. The leaves became more cupped and rugose, the chlorosis developed between the veins, and, in extreme cases, entire areas of the leaf became necrotic. The tendrils developed transverse cracks and later died back from the tips. Death of the terminal bud, a well-recognized symptom of boron deficiency with many plants, did not occur until the leaf and tendril symptoms were quite advanced; finally, however, the terminal bud died and abscissed.

The apical internodes of the deficient vines were much shorter than those of the normal vines. All of the affected parts exhibited abnormal rigidity and brittleness. Young leaves appearing after the onset of the deficiency were often misshapen and malformed, and deeply-lobed with a cut-leaf effect. Deficiency symptoms did not develop on the lower leaves of the vine, even though the terminal parts were extremely affected. On rapidly growing

shoots of the grape, the fifth or sixth leaf from the tip was the oldest leaf to show boron deficiency and, on these, symptoms were usually confined to marginal chlorosis only. This is illustrated in figure 1, which shows the entire growth produced by a minus-boron Lindley vine. The three lower leaves showed no evidence of deficiency symptoms even though the extreme symptoms at the tip had developed a month before the photograph was taken.

FIG. 1. A Lindley grape vine grown under minus boron nutrition in sand culture. Note that the lower leaves show no evidence of deficiency even though growth had ceased and terminal symptoms were severe a month before the photograph was taken.

Apparently the symptoms are developed only in those parts which are in active meristematic condition at the time of occurrence of the deficiency.

EFFECT OF ALTERNATING NUTRIENT SOLUTIONS

The vines which were changed from plus-boron to minus-boron culture and those changed from minus-boron to plus-boron will be referred to respectively as plus-minus and minus-plus boron vines. The minus-boron Golden Muscat and Catawba vines showed extreme symptoms at the time of change. Ten days after application of boron these vines gave evidence of recovery and in fifteen to twenty days the new growth was developing normally. Since the terminal bud had abscissed on these varieties, several lateral buds were forced, giving a bushy appearance to the canes when growth was resumed. A similar condition has been noted in the vineyard (9) and termed "witch's broom." The Herbert vines showed only the first stages of deficiency at the time of changing from minus to plus boron culture. The terminal buds of these varieties resumed growth. In all instances leaves of the minus-plus vines which showed deficiency at the time of changing to the plus-

boron solution retained all of those symptoms even though the new leaves were normal in growth and appearance. The occurrence of deficiency symptoms on the older leaves of a vine therefore would not necessarily indicate a boron deficiency existent in the vine at the time of observation.

The plus-minus boron vines were much slower in developing evidence of deficiency than the minus-plus vines were in recovering from the deficiency. The plus-minus Golden Muscat vines showed tendril necrosis twenty-five days after the change of treatment. A week later the symptoms had progressed to an advanced stage. The Catawba and Herbert plus-minus vines were even slower in exhibiting evidence of the deficiency, the first symptoms appearing in thirty days.

GROWTH OF VINES

Weekly measurement of the canes of each vine throughout the course of the experiment gave an opportunity to correlate linear growth with development of boron deficiency symptoms in the several treatments and varieties. Since a deficiency of boron affected primarily the apical meristematic areas, it is quite evident that the deficient vines should show a distinct curtailment of linear growth. The linear growth of the vines and the weight of the growth produced are given in table I. These data show the much greater growth of the vines in the plus-boron culture, and the effect on the total growth caused by the alternating conditions of boron nutrition. The shoot growth and weight of the minus-plus vines indicate clearly the recovery of the vines after boron was added to the culture solution. Conversely, the growth of the plus-minus vines shows the depressing effect of the omission of boron from the culture solution during the last month of the test.

Since it is of importance to recognize the earliest exhibition of a nutrient deficiency rather than the extreme symptoms, it is of interest to know if the growth rate of the canes of the vines was affected prior to the appearance of definite observable symptoms. Such a condition was observed (data not presented) in the case of the Golden Muscat and Lindley vines, in which there was a distinctly lower growth rate for one or two weeks before any foliar symptoms were apparent.

BORON CONTENT OF THE VINES

The boron content, expressed in parts per million on a dry weight basis, of all parts of all varieties receiving boron in the nutrient solution was much higher than that of comparable parts of vines not receiving boron (table II). This difference was especially pronounced in the concentration of boron in the leaves, with the plus-boron leaves having three to six times as much boron as the minus-boron leaves. The plus-boron vines showed a much greater concentration of boron in the leaves than in the subtending stems. In the minus-boron vines the differences in boron content between the leaves and stems was insignificant, indicating an equalization of concentration in the parts of the boron deficient plants. The boron content of the plus-boron vines ranged from 56.8 to 146.0 p.p.m. in the leaves and from 29.0 to 41.4

TABLE I

LENGTH AND WEIGHT OF SHOOT GROWTH PRODUCED BY TWO-YEAR-OLD GRAPE VINES DURING THREE MONTHS' SAND CULTURE IN THE GREENHOUSE

TREATMENT	HERBERT			CATAWBA			GOLDEN MUSCAT			LINDLEY		
	SHOOT LENGTH	GREEN WT.	DRY WT.	SHOOT LENGTH	GREEN WT.	DRY WT.	SHOOT LENGTH	GREEN WT.	DRY WT.	SHOOT LENGTH	GREEN WT.	DRY WT.
	cm.	gm.	gm.	cm.	gm.	gm.	cm.	gm.	gm.	cm.	gm.	gm.
Continuous plus-boron, March 23–June 23	383	126.4	31.1	371	157.9	37.9	324	128.9	33.2	424	135.4	35.3
Continuous minus-boron, March 23–June 23	201	85.1	24.0	171	73.5	22.3	125	68.8	14.3	140	46.8	11.0
Plus-boron to May 21,* minus-boron thereafter	266	109.0	25.5	325	116.1	28.8	241	113.4	28.5			
Minus-boron to May 21,* plus-boron thereafter	393	120.8	26.0	246	108.5	24.4	218	107.4	19.8			

* Golden Muscat vines were changed in treatment on May 13.

p.p.m. in the stems. The boron content of the minus-boron plants ranged from 10.8 to 27.0 p.p.m. in the leaves and from 20.4 to 23.9 p.p.m. in the stems. It should be pointed out that the lower and upper leaves of the minus-boron Catawba and Herbert vines had a similar boron concentration, although the lower leaves were entirely normal in appearance while the upper leaves had exhibited advanced stages of boron deficiency for a prolonged period previous to sampling.

The boron content of those vines in which there was an alternation of boron nutrition is also given in table II. The withdrawal of boron from the culture solution resulted in greatly decreased boron content of both upper and lower leaves, when compared to the continuous plus-boron culture. Here

TABLE II

THE BORON CONTENT OF GRAPE VINES AFTER THREE MONTHS' GROWTH
IN GREENHOUSE SAND CULTURE

TREATMENT	PORTION OF VINE ANALYZED	BORON ON OVEN-DRY WT. BASIS			
		CATAWBA	HERBERT	GOLDEN MUSCAT	LINDLEY
		p.p.m.	p.p.m.	p.p.m.	p.p.m.
Continuous plus-boron,	Lower leaves	77.4	119.6	123.3	87.8
March 23–June 23	Upper leaves	93.3	146.0	56.8	109.2
	Lower stem	29.3	29.0		
	Upper stem	41.1	41.4		
Continuous minus-boron,	Lower leaves	27.0	24.4	12.7	21.6
March 23–June 23	Upper leaves	23.4	23.6	15.8	10.8
	Lower stem	22.6	20.4		
	Upper stem	21.6	23.9		
Plus-boron until May 21,	Lower leaves	35.8	35.8	42.2	
minus boron thereafter*	Upper leaves	26.3	31.7	16.9	
	Lower stem	39.0	25.0		
	Upper stem	44.1	25.2		
Minus-boron until May 21,	Lower leaves	152.4	90.9	46.3	
plus-boron thereafter*	Upper leaves	60.6	105.3	45.3	
	Lower stem	41.1	28.3		
	Upper stem	65.8	47.3		

* Golden Muscat vines changed in treatment May 13.

again, there is apparently a tendency toward equalization of boron concentration in the various plant parts under conditions of a low boron nutrition level. With addition of boron to the culture solution, the boron level of the minus-plus vines closely approached or even exceeded that of the continuous plus-boron vines at the end of the test. It will be noted that the lower leaves and stems of the minus-plus vines had a boron content greatly in excess of that in the continuous minus-boron vines, indicating a build-up of boron in these tissues after the inception of the plus-boron nutrition.

Thus far, only comparative boron levels of the several treatments at the end of the experiment have been presented. Since with Catawba and Herbert, canes were removed for sampling at the time of change of treatment from plus to minus boron nutrition and vice versa, there is opportunity to

compare the boron content of these plants at the time of change and a month later at which time the effects of the change of treatment were apparent in the foliar development. The differences in boron level of the leaves and stems brought about by the alternation of boron nutrition is shown graphi-
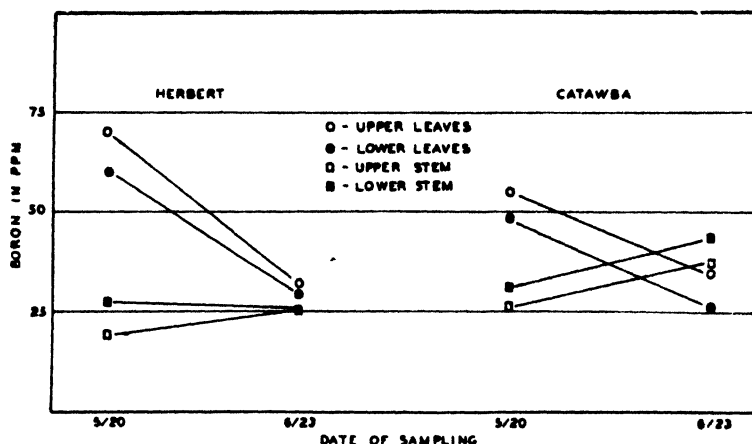
FIG. 2. Effect of changing from plus boron to minus-boron nutrition upon the boron content of shoots of the grape. Treatments changed May 20th.

cally in figures 2 and 3. In general the previous data are substantiated. The change from a plus-boron to a minus-boron nutrient culture resulted in a much lower boron content of both lower and upper leaves although the stems showed slight change in boron level. The addition of boron to the
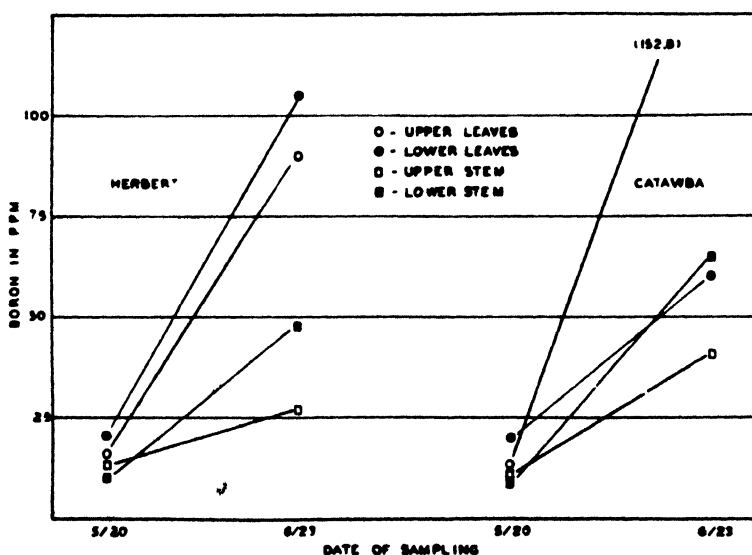
FIG. 3. Effect of changing from minus-boron to plus-boron nutrition upon the boron content of shoots of the grape. Treatments changed May 20th.

nutrient solution caused a great increase in the boron level of the vines grown previously in a minus-boron culture, with the highest level attained in the leaves.

Discussion

The data on growth measurement and boron content of the grape vines grown in the sand culture experiments contribute information on two problems of boron nutrition of plants concerning which there is found controversial or inconclusive statements in the literature. These problems involve: (a) the relationship between boron content of the plant and deficiency symptoms; and (b) consideration of the mobility or retranslocation of boron within the plant.

It has been found that plants exhibit luxury consumption of boron in the presence of an adequate supply, building up a content far above any critical level (8). Also BATJER (1) has found that the difference in boron content between normal and boron-deficient apple fruits may be very slight. Such findings and other factors present difficulties in an attempt to establish a definite relationship between the boron content of the plant and apparent deficiency symptoms. It is clear that in a boron-deficient grape vine the basal leaves, which are normal to all outward appearances, may be just as low or lower in boron than the upper leaves which may show extreme symptoms. Likewise, leaves with extreme boron symptoms may have a boron content equal to that of normal leaves if samples are analyzed following a period of adequate boron nutrition. Such apparently contradictory results are logical if it is assumed that only those leaves which are in actively meristematic condition can develop deficiency symptoms; that boron deficiency symptoms in mature leaves does not disappear when boron nutrition is resumed, although the leaves may increase greatly in boron content.

The above findings are pertinent to the problem of sampling and analysis of plants for the purpose of diagnosing boron deficiencies in the field. The boron content of mature leaves showing deficiency symptoms may in no wise indicate a below-minimum boron level, if the plant had had access to an adequate supply of boron just previous to sampling. The seasonal nature of the occurrence of boron-deficiency symptoms under vineyard conditions shown by the author (10) presents a situation regarding boron nutrition of the vine not dissimilar to that established in the alternation of boron nutrition in sand cultures. Thus there were found in the vineyard, shoots of the current season's growth exhibiting symptoms of boron deficiency on the basal and terminal leaves but with normal leaves in the medial portion.

The definite tendency for the vines to exhibit equalization of boron concentration under low-boron nutrition may possibly serve as a diagnostic index in the attempts to correlate deficiency symptoms with boron levels in the plant.

There seems to be a rather general agreement in the literature concerning the immobility of boron within the plant tissue and the subsequent need of a continuous supply furnished by the nutrient substrate for the maintenance of growth (3, 7, 13, 15). SHIVE (11) concluded that plants of tobacco and cotton cannot build up an available reserve of boron in their tissues which will sustain normal development during a later deficiency of this element.

However, SHIVE in a later paper (12); gives evidence of a portion of the boron within the plant existing in a mobile state by finding 28.7% of the boron in *Vicia faba* and 78.4% of the boron in the corn plant in the expressed sap of the tissues.

Certain of the results in the present experiment can be used as evidence concerning the mobility and transfer of boron in the current season's growth of the grape vine. The growth habit of the vine makes the grape peculiarly adaptable for such studies. In so far as the initial growth of the vines in the minus-boron treatment is concerned, it is impossible to definitely ascertain whether this growth utilized reserve boron in the roots and stem of the two-year-old vine or whether some boron was available in the sand culture media. However, it seems unlikely that the sand, after being thoroughly washed and leached at the beginning of the experiment, would provide available boron for the heavy initial growth which occurred for about thirty days before symptoms of deficiency were observed. Other workers have noted similar results. WEINBERGER and CULLINAN (14) working with one-year-old peach trees cut back to a single bud, obtained normal growth for a period of about six weeks in a minus-boron sand culture. JOHNSTON and FISHER (5) found that tomato plants upon removal to a minus-boron nutrient water culture, continued in growth for a period of about three weeks. It must be assumed that this growth necessitated the utilization of boron present in tissues laid down before the change in nutrient conditions was effected.

The relatively delayed appearance of deficiency symptoms after changing from a plus-boron to a minus-boron nutrition in the present experiment also suggests that there was utilization and transfer of reserve boron. It is interesting, and perhaps suggestive, that the period from the time of withholding boron from the nutrient solution until the appearance of deficiency symptoms, closely approximated the period from growth inception after planting until the initial symptoms of deficiency. Furthermore, the actual amount of cane growth made by the vines during these two periods was not greatly different.

More definite evidence of boron transfer within the vine is afforded by the analyses of the different parts of the vines of the several treatments. In all instances there was a definite reduction in boron content of the lower leaves on the canes of vines which were changed from plus-boron to minus-boron nutrition. Since these leaves were mature and inactive in growth at the time of change of treatment, their greatly lowered boron level could be explained only by assuming a transfer of boron to the upper and active meristematic regions. Since the stems of the plus-boron vines were as high in boron as the stems of the continuous boron vines, it follows that the leaves served largely for storage of reserve boron. This also would be assumed from the fact that the leaves of the vines built up a much higher content of boron under plus-boron nutrition than did the stems.

The apparent equalization of the boron level in various parts of boron-deficient vines of the plus-minus series may also be accounted for on the basis of transfer, since it was shown that a differential existed in these plants

before the deficiency developed. Although there is clear evidence that transfer of boron does occur, and that therefore it can be assumed that the boron in the grape vine is in a mobile state, it is likewise clear that transfer from the lower leaves can take place only when the boron content in these leaves is above a certain level. Boron present in the leaves at this critical level, which was about the same for the three varieties, presumably may be considered to exist in an immobile state insofar as transfer, adequate to maintain normal growth at the growing point, is concerned. Calculated on the basis of analyses of the continuous plus-boron vines, about two-thirds of the total boron in the lower leaves of Herbert and Catawba, and about half of that in Catawba, was transferred during the thirty-day period of minus-boron nutrition in the plus-minus treatment. Of course, the percentage of the total boron present in the mobile state would be greatly dependent upon the degree of "luxury" consumption and reserve accumulation of boron that has taken place.

Study of boron transfer within the plant by analysis of the plant parts was also made by BRANDENBURG (2) with the sugar beet. He found that when the boron supply was interrupted, the new leaves of the sugar beet were very low in boron content; the older leaves, formed while the boron supply was plentiful, retained the normal amount of boron. This result is in direct opposition to the present findings with the grape, in which the older leaves lose boron as the younger leaves are developed under minus-boron nutrition. It is conceivable, as suggested by EATON and BLAIR (4), that the organic form of boron in the tissue of different species of plants may be greatly different, resulting in a widely varying percentage of the boron present in soluble or mobile form.

From the data presented herein on the analyses of the plant parts and on the growth of the vines in the several treatments, it seems logical to conclude that there was a definite transfer of boron from the lower mature leaves on the cane of the grape upward to the terminal actively growing parts, and that the amount thus transferred was sufficient to maintain normal growth of the cane for a limited period, or until the lower leaves were depleted of boron to a certain level. With regard to the findings of other workers on the question of mobility of boron within the plant, it would seem more accurate to interpret their results on a quantitative basis. It is undoubtedly true that a plant cannot store sufficient boron to maintain normal growth for an indefinite period, but this is equally true of other plant nutrients. The conclusion of JOHNSTON and FISHER (5) to the effect that the boron in the plant cannot be used over again and, therefore, must not be considered in the nature of a catalytic agent in plant growth, must be accepted. Their conclusion, however, that boron is fixed in an immobile state in the plant definitely does not hold true for the grape.

Summary

1. Grape vines grown under minus-boron sand culture nutrition in the greenhouse, developed boron deficiency symptoms thirty days after inception

of growth. These symptoms were similar to those previously found under field conditions.

2. Boron-deficient vines resumed normal growth within ten days after changing to plus-boron nutrition. Normal vines changed to minus-boron nutrition continued to grow normally for 25 to 30 days before symptoms of the deficiency were apparent.

3. Only those leaves that were in a meristematic condition at the time of development of the deficiency showed deficiency symptoms. The older basal leaves on boron deficient vines remained normal in appearance even though the terminal leaves exhibited extreme symptoms.

4. In plus-boron vines the boron content of the leaves ranged from 57 to 146 p.p.m. and that of the stems from 29 to 41 p.p.m. In minus-boron vines the concentration of boron was more or less equalized in all parts of the vines at levels of approximately 20 to 25 p.p.m.

5. Vines changed from plus-boron to minus-boron nutrition showed definite lowering of the boron content in the lower mature leaves on the shoot to a point approaching the boron level of continuous minus-boron vines. Coincident with the decrease in boron in the lower leaves was the continued growth in length of the shoots for about 30 days after change of nutrition.

6. A discussion is presented concerning the problem of mobility and retranslocation of boron within plant tissues.

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SOME EFFECTS OF POTASSIUM DEFICIENCY ON THE NITROGEN METABOLISM AND OIL SYNTHESIS IN THE TUNG TREE (*ALEURITES FORDII*)

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It is noteworthy that the great amount of study devoted to the element potassium has revealed but little definite information as to its specific function in plants. One of the difficulties in assigning a definite role to potassium in plant metabolism is the fact that potassium is not known to occur in any definite organic compounds except salts of organic acids and proteins.

MILLER (4) has reviewed the work on the physiological role of potassium in plants, showing that the literature contains many conflicting reports. There appears to be no agreement on the function of potassium in either carbohydrate or protein metabolism. Some investigators, particularly the English workers (3, 9), have emphasized the importance of potassium in the formation of carbohydrates while others (6, 13) have reported that a deficiency of potassium results in an accumulation of carbohydrates. NIGHTINGALE (5) and WALL (13) have summarized the various investigations on the effect of potassium on nitrogen metabolism and, on the basis of their own work as well as that of others, concluded that potassium plays a prominent part in protein metabolism although adequate evidence of its specific function in such processes has not yet been obtained.

Potassium deficiency of the tung tree (*Aleurites fordii*), widespread in northern Florida and southern Georgia, is one of the major nutritional problems of that important tung-growing area (1). Preliminary field trials in a 4-year-old orchard have shown that the application of potassium fertilizers corrected the symptoms of interveinal chlorosis and necrosis associated with potassium deficiency (7). These plots provided an opportunity for investigating the effects of potassium deficiency on the metabolism of the tree, particularly with regard to oil formation. The effects of a supplementary nitrogen treatment were also investigated.

Materials and methods

In August 1941, 4-year-old trees were selected in a potassium-deficient area near Capps, Florida, and grouped on the basis of the severity of potassium-deficiency symptoms so that comparable plots were obtained for each treatment (7). Each of the following treatments was applied in the spring of 1942 to each of six single-tree plots and was supplementary to a blanket

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application of 4 pounds per tree of a 3–6–7 mixed fertilizer: (1) none, (2) five pounds of potassium chloride, (3) four pounds of sodium nitrate, and (4) three pounds of potassium nitrate. On each of three sampling dates, two composite leaf samples were taken from the trees of each treatment, one from replications 1, 2, and 3, and another from replications 4, 5, and 6. In compositing the leaf samples, 25 midshoot leaves were taken from each tree, thus making a total of 75 leaves per sample. Analogous composite fruit samples comprising five fruits from each tree were taken in the same manner and at the same time. Both leaf and fruit samples were obtained on June 26,[2] August 13, and October 21 to correspond with the respective periods (I) just preceding synthesis of oil in the kernels, (II) midpoint of active oil formation, and (III) maturation of the kernel, as indicated by previous studies (11).

The composite samples of leaves and of fruit were prepared and the analyses made essentially as described by SELL et al. (11) with the modifications introduced by GILBERT, SELL, and DROSDOFF (2). In the latter paper the methods are given for determining the various nitrogen fractions. The following determinations were made on both the leaf and fruit samples: dry matter, reducing sugar, non-reducing sugar, starch, acid-hydrolyzable polysaccharides other than starch, potassium, and total insoluble amide, and amino nitrogen. As the insoluble nitrogen is largely protein nitrogen, this term will be used for convenience in referring to the insoluble nitrogen fraction. In addition, oil in the kernels and the percentage of kernels in the whole fruit were determined.

Results

LEAVES

Data on leaf composition are given (table I). In all tables each value represents the average of the analyses of two composite samples. Since the original determinations were made in duplicate, the statistical significance of the mean differences between treatments and of the interaction of treatments with the sampling date was determined. Although these data were also calculated on a per plant-part basis, the data thus obtained showed no important trends other than those indicated by the percentage data. Consequently, the data on the per plant-part basis are not presented here.

First it should be noted that the potassium content was considerably higher throughout the season in the leaves of the potassium-treated trees than in those not treated with potassium. These differences had high statistical significance. Also the nitrogen treatment resulted in a highly significant increase in nitrogen content. This shows that fertilizer treatments were effective and that the concomitant changes in the organic reserves of the plant are associated with increased potassium and nitrogen due to treatment.

Leaf samples collected in June from the potassium-deficient trees showed

[2] Trees treated with 4 pounds of sodium nitrate were not sampled on June 26.

TABLE I

COMPOSITION OF LEAVES FROM UNTREATED AND POTASSIUM- AND NITROGEN-TREATED 4-YEAR-OLD TUNG TREES IN A POTASSIUM-DEFICIENT AREA NEAR CAPPS, FLORIDA, 1942

SAMPLING DATE	TREATMENT†	DRY MATTER	REDUCING SUGAR	NON-REDUCING SUGAR	STARCH	POLYSACCHA-RIDES OTHER THAN STARCH	TOTAL N	PROTEIN N	AMIDE N	AMINO N	K
		%	%	%	%	%	%	%	%	%	%
June 26	Check	46.8	4.50	4.36	4.70	4.42	1.92	1.80	0.03	0.08	0.67
	K	41.4	5.44	3.50	2.86	3.80	2.20	2.08	0.03	0.09	1.12
	KN	44.6	4.88	5.28	2.25	3.46	2.60	2.48	0.04	0.09	0.95
Aug. 13	Check	50.8	4.71	4.88	1.74	5.36	1.84	1.72	0.04	0.08	0.56
	K	46.1	4.98	4.12	3.34	5.44	1.86	1.74	0.03	0.08	0.85
	N	52.7	4.20	6.45	1.31	5.50	2.41	2.28	0.04	0.10	0.48
	KN	45.9	5.60	5.46	2.42	5.84	2.40	2.26	0.04	0.12	0.87
Oct. 21	Check	53.0	4.24	3.66	0.42	4.12	1.83	1.68	0.03	0.12	0.54
	K	48.4	4.72	3.84	0.68	3.70	1.88	1.71	0.03	0.13	0.91
	N	52.3	3.45	4.18	0.54	4.27	2.23	2.04	0.03	0.16	0.46
	KN	50.0	5.00	4.98	0.54	4.12	2.18	1.98	0.04	0.18	0.84

CONSTITUENTS*

* Average of two composite samples, moisture-free basis.
† All trees received 4 lbs. per tree of a 3-6-7 fertilizer in March supplemented by the following treatments: Check = no additional fertilizer; K = 5 lbs. of KCl; N = 4 lbs. of $NaNO_3$; KN = 3 lbs. of KNO_3.

more starch and polysaccharides than those from potassium-fertilized trees. No visible deficiency symptoms were apparent at that time. By the August sampling date, however, the starch in the check trees had decreased markedly, reaching a level even lower than the earlier level of the potassium-treated trees which meanwhile had increased in starch (though this increase was not statistically significant). The higher starch content of the potash-treated trees in August had high statistical significance. At this time the abnormal leaf pattern due to potash deficiency was evident in both the check and nitrogen-only treated trees. This coincides with the period when both translocation of materials from the leaves to the fruit and oil formation are proceeding at a rapid rate. It is noted also that the nitrogen treatments effected a highly significant increase in non-reducing sugar at the August and October sampling dates.

Most of the nitrogen in the leaves is present in protein form, the amide and amino fractions being present only in small amounts. Tests for ammonium and nitrate nitrogen were negative. Therefore the nitrogen treatment primarily had increased the protein content of the leaves, although the increase in amino nitrogen attained high statistical significance. The potassium treatment is associated with an increased protein content of the leaves over the check at the time of the first sampling, but not in the later periods.

FRUIT AND ITS COMPONENT PARTS

Data are presented on the analysis of the kernels (table II), and on the analysis of the hulls (table III). These data together with that on the analysis of the shells were combined to give the composition of the fruit as a whole (table IV). Shells were included with the kernel fraction at the first sampling period because of difficulty of mechanical separation at that stage. At other periods the shells were removed from the kernels and analyzed separately. Since composition of the shells was found to be quite similar to that of the hulls, data for this fraction are not presented except as included in the calculation of the composition of the whole fruit.

At the time of the first sampling in the latter part of June there was a highly significant increase in the percentage of potassium in the fruit, particularly in the hulls, due to potassium treatment. The nitrogen treatments increased the nitrogen content of the fruit, but to a lesser extent. Nitrogen treatments also resulted in a highly significant increase in the protein content of both kernels and hulls and in the amino nitrogen content of the hulls. At this date the polysaccharides in the kernels from fruit of potassium-plus-nitrogen-treated trees were higher than those from trees receiving other treatments. However, the polysaccharide content of the hulls was highest in fruit from untreated trees. No significant differences due to treatment were found in the other carbohydrate fractions.

At the time of the August sampling, oil formation was well under way and the effect of the potassium treatments on increasing the oil content of the kernels and the whole fruit was apparent. The increased percentage of

TABLE II

Composition of Kernels from Untreated and Potassium- and Nitrogen-Treated 4-Year-Old Tung Trees in a Potassium-Deficient Area near Capps, Florida, 1942

Sampling Date	Treatment[†]	Constituents[*]										
		Dry Matter	Reducing Sugar	Non-Reducing Sugar	Starch	Polysaccharides Other Than Starch	Total N	Protein N	Amide N	Amino N	K	Oil
		%	%	%	%	%	%	%	%	%	%	%
June 26	Check	9.98	3.68	6.66	2.18	7.43	1.61	1.07	0.19	0.36	1.18	
	K	9.60	3.74	6.14	1.88	7.96	1.68	1.12	0.20	0.37	1.30	
	KN	10.70	2.80	4.88	1.46	10.57	1.82	1.18	0.23	0.40	1.52	
Aug. 13	Check	22.32	Trace	2.72	Trace	4.28	3.66	2.27	0.24	1.15	0.86	30.9
	K	21.42	Trace	4.62	Trace	4.37	3.50	2.06	0.28	1.16	0.88	32.7
	N	23.20	Trace	2.56	Trace	4.28	3.79	2.27	0.26	1.26	0.68	28.6
	KN	30.18	Trace	2.22	Trace	3.78	3.72	2.40	0.28	1.04	0.86	33.3
Oct. 21	Check	96.40	0.77	11.31	Trace	8.88	2.22	1.75	Trace	0.48	0.62	62.3
	K	98.32	0.72	8.76	Trace	8.42	2.11	1.60	Trace	0.50	0.54	65.4
	N	98.51	0.76	5.94	Trace	6.80	2.86	2.24	Trace	0.62	0.63	62.4
	KN	98.55	0.23	5.72	Trace	7.12	2.75	2.32	Trace	0.44	0.58	65.4

* Average of two composite samples, moisture-free basis.

† All trees received 4 lbs. per tree of a 3-6-7 fertilizer in March supplemented by the following treatments: Check = no additional fertilizer; K = 5 lbs. of KCl; N = 4 lbs. of NaNO₃; KN = 3 lbs. of KNO₃.

TABLE III

COMPOSITION OF HULLS FROM UNTREATED AND POTASSIUM AND NITROGEN-TREATED 4-YEAR-OLD TUNG TREES IN A POTASSIUM-DEFICIENT AREA NEAR CAPPS, FLORIDA, 1942

SAMPLING DATE	TREAT-MENT[†]	DRY MATTER	REDUCING SUGAR	NON-REDUCING SUGAR	STARCH	POLYSAC-CHARIDES OTHER THAN STARCH	TOTAL N	PROTEIN N	AMIDE N	AMINO N	K
		%	%	%	%	%	%	%	%	%	%
June 26	Check	24.10	3.54	4.20	0.60	11.63	0.83	0.74	0.04	0.05	0.70
	K	21.92	4.80	4.13	0.74	9.47	0.88	0.76	0.02	0.10	1.27
	KN	22.90	3.66	3.28	0.89	9.24	1.14	0.95	0.05	0.13	1.65
Aug. 13	Check	25.14	2.90	3.54	0.36	8.17	0.59	0.48	0.03	0.08	1.08
	K	22.92	2.98	3.63	0.27	6.74	0.52	0.44	0.02	0.07	1.84
	N	25.76	3.79	3.68	0.29	6.95	0.90	0.68	0.06	0.15	0.95
	KN	24.48	3.48	3.39	0.26	7.14	0.78	0.62	0.03	0.13	1.80
Oct. 21	Check	77.8	0.32	Trace	0.66	9.09	0.31	0.31	Trace	Trace	1.96
	K	83.2	0.68	Trace	0.51	8.13	0.27	0.27	Trace	Trace	2.67
	N	83.0	0.80	Trace	0.58	8.80	0.44	0.44	Trace	Trace	1.45
	KN	84.4	0.38	Trace	0.52	7.30	0.42	0.42	Trace	Trace	3.08

CONSTITUENTS*

* Average of two composite samples, moisture-free basis.

† All trees received 4 lbs. per tree of a 3–6–7 fertilizer in March supplemented by the following treatments: Check = no additional fertilizer; K = 5 lbs. of KCl; N = 4 lbs. of NaNO₃; KN = 3 lbs. of KNO₃.

TABLE IV

COMPOSITION OF WHOLE TUNG FRUIT FROM UNTREATED AND POTASSIUM- AND NITROGEN-TREATED 4-YEAR-OLD TUNG TREES IN A POTASSIUM-DEFICIENT AREA NEAR CAPPS, FLORIDA, 1942

SAMPLING DATE	TREATMENT†	CONSTITUENTS*											
		DRY MATTER	REDUCING SUGAR	NON-REDUCING SUGAR	STARCH	POLYSACCHARIDES OTHER THAN STARCH	TOTAL N	PROTEIN N	AMIDE N	AMINO N	K	KERNELS	OIL
		%	%	%	%	%	%	%	%	%	%	%	%
June 26	Check	19.10	3.56	4.64	0.90	10.86	0.97	0.80	0.07	0.11	0.79	18.4	....
	K	17.72	4.60	4.50	0.95	9.20	1.02	0.82	0.06	0.14	1.28	18.5	....
	KN	18.53	3.48	3.60	1.01	9.51	1.28	1.00	0.08	0.20	1.62	20.7	....
Aug. 13	Check	27.97	2.18	2.95	0.26	8.06	0.84	0.62	0.05	0.18	0.88	9.2	2.88
	K	26.55	2.04	2.94	0.18	7.04	0.80	0.58	0.05	0.17	1.34	10.2	3.42
	N	28.92	2.72	2.95	0.20	7.33	1.11	0.78	0.08	0.25	0.76	10.2	2.96
	KN	29.34	2.32	2.56	0.16	7.22	1.14	0.82	0.07	0.25	1.32	14.7	4.90
Oct. 21	Check	84.10	0.42	3.52	0.38	8.79	0.89	0.75	Trace	0.14	1.12	30.2	18.84
	K	87.96	0.60	2.92	0.26	8.25	0.90	0.72	Trace	0.17	1.31	34.4	22.50
	N	87.78	0.64	2.00	0.31	8.13	1.26	1.05	Trace	0.21	0.76	34.1	21.29
	KN	88.82	0.28	2.10	0.27	7.54	1.30	1.14	Trace	0.16	1.44	37.6	24.62

* Average of two composite samples, moisture-free basis.

† All trees received 4 lbs. per tree of 3–6–7 fertilizer in March supplemented by the following treatments: Check = no additional fertilizer; K = 5 lbs. of KC N = 4 lbs. of NaNO₃; KN = 3 lbs. of KNO₃.

oil in the whole fruit resulted from an increase in the percentage of both oil in the kernel and of kernel in the fruit. The increased potassium content of the hulls (and consequently of the whole fruit) resulting from potassium treatment, was even more marked at this stage than at the first sampling. Carbohydrate fractions at this time showed no significant differences due to treatment. As at the time of the first sampling, protein content of the fruit was higher in the nitrogen-treated trees, and this increase had high statistical significance. Other nitrogen fractions showed no consistent differences due to treatment.

The effect of potassium treatments in increasing the oil content of the fruit was pronounced at the last sampling date with fully mature fruit. The increase in the percentage of oil in the kernels and in the percentage of kernels in the whole fruit from trees receiving the potassium treatments was highly significant statistically. The nitrogen treatments resulted in a highly significant increase in the protein content of both the kernels and the hulls and in the amino-nitrogen content of the hulls. Kernels from the untreated trees were high in non-reducing sugar which had not been converted to oil or protein. The potassium treatment without nitrogen lowered the sucrose content and increased the oil in the kernels but not the protein, in comparison with the check treatment. The nitrogen-alone treatment, on the other hand, did not significantly affect the oil content of the kernels but increased the amount of protein present, thus accounting for the lower sucrose content in comparison with the kernels from the check treatment. The potassium-plus-nitrogen treatment produced kernels with high oil, high protein, and low sucrose content.

The potassium treatments markedly increased the potassium content of the hulls but not of the kernels. Actually there was a trend toward a lower potassium content in the kernels as a result of potassium treatment, but this decrease did not attain statistical significance.

Discussion

The pronounced effect of potassium deficiency on the composition of tung fruit is the most striking observation in this investigation. The effect was clearly evidenced by the middle of August when oil formation was well under way. At that time the potassium-treated trees showed a higher percentage of oil in the kernel and a higher percentage of kernel than did the check trees. No significant differences in carbohydrate content were associated with any treatments at this stage. In the mature fruit, however, the kernels from the check trees showed an accumulation of sucrose, and this was associated with a lower oil content compared to that of the potassium-treated trees.

Since it has been shown that sucrose (10) is the most important carbohydrate reserve utilized in oil formation in mature tung kernels (11), it is logical to assume that this constituent may be considered as the storage form alternate to the oil and protein in the seed, accumulating when formation

of the other two reserves is checked. This suggests that potassium may function in the process of conversion of sugar into oil. The nitrogen-alone treatment has also resulted in a lower sucrose content than the check treatment, but this was associated with an increased amount of protein, the oil content remaining about the same. Other more extensive experiments with nitrogen fertilization of tung trees (8, 12) have shown that there is actually a decrease in the percentage of oil in the kernel with increased nitrogen fertilization, resulting in kernels with relatively high protein content.

In addition to effecting an increase in the oil content of the kernel, the potassium treatment has also resulted in an increased percentage of kernel in the whole fruit in comparison with the check trees, thereby substantially increasing the oil content of the whole fruit. These data are in agreement with the findings of other investigators (8) and the results of a number of other experiments being conducted at the tung laboratories of the Bureau of Plant Industry, Soils, and Agricultural Engineering of the U. S. Department of Agriculture.

With reference to the data on the leaves it would appear that potassium deficiency shows two stages. The early stage (June sampling), at which no symptoms are apparent, is characterized by an accumulation of starch and polysaccharides in the leaves. At the late stage (August sampling), starch tends to disappear from the leaves of potassium-deficient trees to a greater extent than from the leaves of the potassium-treated trees. At this time there is a rapid translocation of materials from leaves to fruit. This physiological pattern of potassium effects is similar to that found in other plants (5, 14).

Summary

During the growing season the composition of leaves and fruit of 4-year-old tung trees low in nitrogen and potassium was compared with that of trees fertilized with these two elements. From these data the following conclusions have been drawn:

1. The leaf data indicate that potassium-deficient trees show two stages; early in the season starch and polysaccharides accumulate, while later in the season they tend to disappear to a greater extent than from potassium-treated trees.

2. Fruit from potassium-treated trees had a higher oil content than fruit from untreated trees, due both to a higher percentage of oil in the kernel and to a higher percentage of kernel. The low oil content and the accumulation of sucrose in kernels of potassium-deficient trees suggest that potassium may function in the process of conversion of sugar into oil.

3. Fruit from nitrogen-treated trees had a higher percentage of kernel and a higher protein content in the kernel than fruit from untreated trees.

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VITAMIN STUDIES OF VARIETIES AND STRAINS OF PEAS

P. H. Heinze, Frances R. Hayden, and B. L. Wade

The importance of garden peas in the diet of the population of this country is evidenced by the increasing volume of peas that are commercially canned and frozen each year. The widespread growing of peas for home use is an additional factor making the study of their nutritive value all the more important.

It has been shown that peas can supply a considerable portion of some of the vitamins in the diet. Fincke et al. (3) pointed out that the thiamine values for 19 varieties ranged from 2 micrograms per gram, on the fresh weight basis, for Laxton's Progress to 7.1 micrograms per gram for World Record. In a study involving six varieties Pepkowitz et al. (8) found statistically significant differences between varieties for content of carotene and of ascorbic acid, but the magnitude of these differences was very small in comparison with other reports for thiamine content. Scott and Belkengren (10) have shown that the carotene content in peas varied greatly, the highest value being over four times the lowest when approximately 78 strains were compared; and that the greatest ascorbic acid content was 2.7 times the lowest. However, in some instances these reported values must be assumed to have been based on a single determination and in other cases varieties harvested in different years or different locations have been compared for varietal variation.

In the present study a number of varieties and strains of peas were grown under as nearly identical field conditions as possible over a period of several years and the variations in vitamin content studied in detail.

Methods

The peas were grown at Charleston, South Carolina in 32-foot rows, randomized blocks, or in a simple lattice with 2 to 4 replications. Approximately one pound of pods was picked at random along the entire length of the row. The peas were brought into the laboratory, shelled, and suitable samples were weighed for the various analyses.

Ascorbic acid was determined according to the method described by Heinze et al. (4). Twentyfive-gram samples were extracted with 1% meta-phosphoric acid and the reduction of 2,6-dichlorophenolindophenol dye was determined with an Evelyn photoelectric colorimeter.

Thiamine was determined by a modification of Conner and Straub's (1) thiochrome procedure. Forty grams of fresh peas were blended with approximately 100 ml. of 0.04 N H_2SO_4 in the small-sized Waring blender container. The mixture was quantitatively transferred to a 250-ml. pyrex

volumetric flask with additional 0.04 N H_2SO_4 and heated in a boiling water bath for 30 minutes. The contents of the flasks were stirred by rotating the flasks every 5 to 10 minutes during the heating period. The flasks were cooled to approximately 40° C. and sufficient sodium acetate–acetic acid buffer was added to bring the contents to pH 4.5 ± 0.1. Enough takadiastase, papain, and Pectinol enzymes were added to the buffer so that the aliquot of buffer placed in each flask would contain 0.1 gm. of each enzyme. The solutions were incubated in a water bath over night (16 hours) at 40° C., made to volume, and filtered. Aliquots of the filtrate were run through the Decalso columns at room temperature and the analysis completed as described by CONNER and STRAUB.

Riboflavin determinations for the 1943 and 1944 harvests were made on the filtrate of the enzyme digest according to the method of MACKINNEY and SUGIHARA (7). In 1945 the determinations were made directly on the filtrate, as described by PETERSON et al. (9). The Coleman Model 12 photofluorometer was used for reading the final solutions for the thiamine and riboflavin determinations.

Carotene analyses were made as described by WALL and KELLEY (12), with some modifications. Instead of adding 100 ml. of 5% sodium sulfate solution during the phasic separation, 100 ml. of 30% KOH was introduced. The KOH broke the emulsions much more rapidly, produced a sharper interface, and also removed nearly all of the chlorophyll through saponification. The final solution was read in an Evelyn photoelectric colorimeter with filter #440.

Moisture determinations were made by drying approximately 30 gm. to constant weight in a forced-draft rapid-drying oven at 70° C.

Results

ASCORBIC ACID CONTENT OF PEAS

In 1942 thirty-three varieties or strains of peas were grown in a randomized block experiment in two replicates. The varieties were harvested at weekly intervals on three different dates in April, making a total of 6 analyses for each variety. Varietal means for ascorbic acid content ranged from 29.0 to 44.4 mg. per 100 grams, on a fresh weight basis, and the differences required for significance were 4.2 mg. and 5.7 mg. at the 5% and 1% levels, respectively. Some of these varieties and strains were also analyzd in 1944 and 1945. Data for the ascorbic acid content of these 18 varieties and strains of peas in 3 different years are presented in table I.

THE EFFECT OF SIZE AND MATURITY ON VITAMIN CONTENT

In May, 1943 eight varieties of peas were harvested on the same day. The shelled peas from all varieties were in prime condition for eating. Screens were used to divide the peas of each variety into three approximately equal lots according to size, small, medium, and large. Various degrees of maturity or stages of development were represented in the three size lots

from each variety. Different screens were used to separate the three lots for the naturally small-seeded varieties, Willet's Wonder and Creole, than were used for the large-seeded varieties such as Progress. All lots of each variety were analyzed for ascorbic acid, thiamine, riboflavin, and moisture content.

In table II means on a fresh and dry weight basis are given for each of the three size lots of peas for all varieties combined. The dry matter contents for the small, medium and large size lots averaged 20.8, 25.6 and 30.5% respectively. On a fresh weight basis significant differences were found

TABLE I

ASCORBIC ACID CONTENT OF PEAS IN DIFFERENT YEARS

VARIETY OR STRAIN*	MG./100 GM., FRESH WEIGHT BASIS		
	1942	1944	1945
Alah	40.7	37.4	
Hundredfold	35.2	34.6	29.9
Progress	34.7	36.2	42.6
Progress × Giant Stride			
762A	36.4	41.6	36.2
899	36.4	39.6	33.4
770–5	35.0	41.1	
900	37.1	37.3	
298–6B–3	29.0	34.2	
898		32.6	33.3
Progress × Kent Alderman			
591–3–1–1	30.7	35.9	32.9
902	37.6	41.7	
Progress × World Record			
557–1–13	31.2	36.5	28.4
772B	34.2		31.9
Little Marvel × (Thomas Laxton × Phenomenon)			
911	36.9	36.2	32.3
781–2B	30.9	33.3	32.4
910		31.0	34.0
Little Marvel × World Record 775–1B	31.5	33.5	
Phenomenon × Thomas Laxton 318–3B	32.6		30.7
L.S.D. 5%	4.2	6.5	3.3
1%	5.7	8.6	4.4

* Numbers represent same strains throughout paper.

between the several lots in both thiamine and ascorbic acid contents, the small size lot averaging the highest ascorbic acid content and the large, the highest thiamine content. No significant variation occurred in the amount of riboflavin present although there was a slight trend toward a decreasing amount with increasing maturity. This trend in riboflavin content is brought out more clearly in the figures based on dry weight where a highly significant difference exists among the three sizes. On a dry weight basis both the riboflavin and ascorbic acid contents rapidly decreased as the peas matured. The observed decrease in ascorbic acid content of peas as they mature has been noted by other workers (2, 5, 6, 8). The thiamine content increased as the dry matter content increased, thus minimizing the differ-

ences between lots for thiamine on the dry weight basis. There was no significant difference in thiamine content between the medium and large size lots on a dry weight basis, but both were significantly lower in thiamine than the small size lot. LEE and WHITCOMBE (5) have also found that thiamine expressed on a fresh weight basis increased with increasing tenderometer readings but remained relatively constant, with the exception of the most mature lot, when expressed on a dry weight basis.

The eight varieties of peas analyzed were of different seed types: Willet's Wonder and Creole represented small-seeded types; Progress and several breeding lines, large-seeded types; while Wando and strain 802–2 were intermediate in seed type. On a fresh weight basis the mean value for

TABLE II

THE RELATION OF SIZE AND PERCENTAGE DRY MATTER TO THE VITAMIN CONTENT OF GARDEN PEAS*

	ASCORBIC ACID	THIAMINE	RIBOFLAVIN	DRY MATTER
FRESH WEIGHT BASIS				
	mg./100 gm.	mcg./100 gm.	mcg./100 gm.	%
Small	41.7	287	131	20.8
Medium	33.0	309	122	25.6
Large	24.7	358	111	30.5
L.S.D. 5%	2.9	33		2.8
1%	4.0	45		3.9
DRY WEIGHT BASIS				
	mg./gm.	mcg./gm.	mcg./gm.	
Small	2.01	13.72	6.34	
Medium	1.32	11.84	4.90	
Large	0.83	11.60	3.69	
L.S.D. 5%	0.18	1.11	0.82	
1%	0.26	1.54	1.13	

* Means of 8 varieties analyzed.

ascorbic acid for all lots from the small-seeded varieties was 34.9 mg. per 100 grams; the intermediate varieties, 34.0; and the large-seeded varieties, 30.4. On a dry weight basis the mean ascorbic acid contents were 1.29, 1.55, and 1.34 mg. per gram, respectively. Because of a higher percentage of dry matter in the small-seeded varieties the slightly higher ascorbic acid value on the fresh weight basis disappeared when the values were expressed on a dry weight basis. Thiamine content appeared to be unrelated to seed type of the different varieties. The highest and the lowest variety means for thiamine content both occurred in the large-seeded types. Riboflavin content does not appear to be related to the seed type although Willet's Wonder, a small seeded variety, averaged the highest riboflavin content.

GENERAL SURVEY FOR THIAMINE, RIBOFLAVIN, AND ASCORBIC ACID CONTENT

In 1943 a survey was made on over 100 varieties or strains for general information concerning the range of content of the three vitamins, thiamine,

riboflavin, and ascorbic acid. These data are given in table III. The strains selected from the various crosses were developed by single-plant selections for 4 to 10 generations and many had been carried through addi-

TABLE III

VITAMIN CONTENT OF VARIETIES AND STRAINS OF PEAS ON FRESH WEIGHT BASIS.
SURVEY 1943

VARIETY OR CROSS	SAMPLES OR STRAINS		THIAMINE	RIBOFLAVIN	ASCORBIC ACID	DRY WEIGHT
	Number		*mcg./100 gm.*	*mcg./100 gm.*	*mg./100 gm.*	*%*
Creole	2		328	119	35.0	25.8
Gilbo	2		285	94	28.6	25.6
Glacier	1		183	97	29.3	22.4
Hundredfold	3		268	95	25.7	22.6
Papago	1		309	112	36.9	26.7
Perfection	2		341	82	32.2	27.0
Perfectah A	2		246	99	36.8	24.2
Perfectah B	3		338	91	31.2	23.5
Perfectah, Early	2		292	90	30.3	23.6
Phenomenon	1		477	105	27.6	25.2
Progress	2		108	103	24.8	22.8
Progress*	2		303	84	34.4	23.5
Stratah	1		357	99	25.8	22.5
Wando	4		285	102	32.2	23.4
Walah	2		330	92	24.3	26.1
Willet's Wonder	5		336	131	39.7	29.2
Progress × Giant Stride	40	range	91–382	78–119	21–43	19–30
		mean	210	93	30.8	22.7
Progress × Kent Alderman	9	range	235–316	76–99	25–35	20–27
		mean	271	92	31.6	22.3
Progress × Perfection	4	range	236–309	97–112	31–39	21–23
		mean	276	106	35.8	21.6
Progress × World Record	4	range	144–234	95–112	25–32	20–25
		mean	194	106	27.2	21.9
Little Marvel × Wisconsin Early Sweet	4	range	242–323	78–93	28–31	26–27
		mean	291	86	29.0	26.0
Little Marvel × World Record	7	range	221–323	76–99	28–34	20–27
		mean	271	92	31.6	22.3
Little Marvel × (Thomas Laxton × Phenomenon)	6	range	257–309	76–99	27–32	23–26
		mean	286	90	29.1	23.9
Giant Stride × Sutton's Excelsior	2	range	366–420	97–101	20–21	22–26
		mean	393	99	20.5	23.9
Giant Stride × World Record	1		273	84	32.5	21.5

* Derived from Progress crosses and now sold as Progress.

tional generations as bulk lines. It was found that the greatest range occurred in the thiamine content, with some varieties giving indications of having 3 to 4 times the quantity of others. The ranges in riboflavin and ascorbic acid content were much smaller than for thiamine; but the two were

of nearly the same relative magnitude, the highest value being approximately twice the lowest value for each vitamin.

The thiamine content of the 40 strains from the cross Progress × Giant Stride ranged from 91 to 382 micrograms per 100 grams on the fresh weight basis. This indicated a considerable segregation for thiamine content. The Progress parent used in these crosses gave a mean value of 108 micrograms. Giant Stride does not thrive under field conditions at Charleston, hence no comparable values could be given but reports in the literature indicate that it ranks high in thiamine content. The two strains from Giant Stride × Sutton's Excelsior were very high in thiamine but were the lowest in ascorbic acid of any of the strains analyzed. The Progress × Perfection crosses gave the highest values for ascorbic acid. The range in riboflavin content among the breeding strains was less than that for ascorbic acid, but the variety Willet's Wonder contained much more than any of the breeding strains. On a fresh weight basis the three small-seeded varieties, Creole, Papago, and Willet's Wonder, had a higher mean ascorbic acid content, 37.2 mg. per 100 grams, than the intermediate types, such as Perfection, the Perfectahs, and Wando with a mean value of 32.5, or the large-seeded varieties with 27.6 mg. per 100 grams. Again these differences in favor of the small-seeded types disappeared when the ascorbic acid values were calculated on a dry weight basis. In this survey work the small-seeded types had slightly higher riboflavin values than the other types when expressed on the fresh weight basis.

Detailed Studies on Thiamine, Ascorbic Acid, Carotene, and Riboflavin Content

In 1944 two experiments were designed to study in detail the thiamine, ascorbic acid, carotene, and riboflavin content of 34 varieties or strains. In the first experiment 22 of the earlier-maturing varieties were used. Six harvests were made over a period of 24 days, with 4 harvests of any one variety extending over 9 or 10 days. There was such a large variation associated with the wide-spread dates of harvest within single varieties, or groups harvested alike, that varietal differences were obscured in the combined harvest data. It was found that the first harvest of the earlier varieties and the last harvest of the later ones were distinctly not typical of the other three harvests of the respective varieties. By dropping the data of these atypical harvests and using only the three typical harvests of each variety, the spread of the harvest dates is reduced to 11 days and no abnormal material is included in the variety data. The means for the three harvests are given in table IV. When the data from these three harvests were analyzed statistically as randomized blocks there was no significant harvest-to-harvest variation except for riboflavin content. Each harvest came from a different field replicate, which caused a confounding of harvests with field replicates. Since the three harvests were not significantly different it may be assumed that field replicates had very little influence on the variation. The lack of influence of field replicates is brought out more

clearly in the experiment with the later varieties. The F values for varie-
ties in the early-maturing group were significant at the 1% level for thia-
mine and carotene and at the 5% level for ascorbic acid and riboflavin.

Six strains from the Progress × Giant Stride cross were represented in
this experiment. The range in thiamine content was from 116 to 352 micro-
grams per 100 gm., strains 899 and 900 having the highest values. These

TABLE IV

VITAMIN CONTENT OF 22 EARLY VARIETIES OR STRAINS OF PEAS. AVERAGE OF 3 HARVESTS.
FRESH WEIGHT BASIS. 1944

VARIETY OR STRAIN	THIAMINE	RIBO-FLAVIN	ASCORBIC ACID	CAROTENE	DRY WEIGHT
	mcg./100 gm.	*mcg./100 gm.*	*mg./100 gm.*	*mcg./100 gm.*	*%*
Alah	275	100	37.4	560	26.8
Glacier	260	92	34.3	406	20.7
Hundrefold	321	91	34.6	471	19.5
Progress	129	86	36.2	474	21.4
Progress × Giant Stride					
298–6B–3	116	87	34.2	455	20.8
762A	221	94	41.6	488	22.6
763–1–1	308	95	35.0	478	19.3
770–5–1	155	93	41.1	451	20.1
899	314	109	39.6	475	19.6
900	352	104	37.3	451	19.6
Little Marvel × World Record					
775–1–B–1	310	107	33.5	492	21.0
776–1B	209	101	38.4	477	21.0
777–1B	241	96	35.7	475	20.9
779B–1–1	283	96	35.0	454	21.4
Little Marvel × (Thomas Lax-ton × Phenomenon)					
781–2B	316	99	33.3	383	22.8
910	322	93	31.0	396	24.1
911	303	117	36.2	425	22.5
Progress × Kent Alderman					
591–3–1–1–1	325	78	35.9	461	21.1
902	370	98	41.7	468	18.7
Progress × World Record					
557–1–13	250	83	36.5	450	21.2
Phenomenon × World Record					
787	320	89	29.8	454	21.7
1–17*	328	91	40.0	444	19.9
L.S.D. 5%	69	17	6.5	43	2.2
1%	93	23	8.6	57	2.9

* Uncertain pedigree.

two strains were among the highest of the 40 strains from this cross analyzed
in 1943. Strain 298–6B–3 had the low value of 116 mcg./100 gm. in this
experiment. In 1943 the same strain had for 3 analyses a mean content of
93 micrograms per 100 gm. The Progress parent was again very low in
thiamine content. Strains 781–2B, 910, and 911 from the cross Little Mar-
vel × (Thomas Laxton × Phenomenon) gave relatively high values for thia-
mine. In 1943 strains from the same cross had a mean thiamine content
greater than all but two other crosses.

As in 1943 the range in riboflavin content was much less than that for thiamine. Very few significant differences occurred among the strains.

The high value of 41.7 mg. per 100 gm. for the ascorbic acid content of strain 902 corresponded to a high of 35 mg. in the 1943 when it was the highest in rank among the 9 strains from the Progress × Kent Alderman cross.

The first data for carotene content in the present study are given in table IV. Although the range in carotene content was not as great as that for other vitamins a highly significant varietal or strain difference was found. This difference was not found between the strains within a cross; but highly significant differences were found between the strains of different crosses. For instance, the strains from the Little Marvel × (Thomas

TABLE V

VITAMIN CONTENT OF 12 LATE VARIETIES OR STRAINS OF PEAS. AVERAGE OF 4 ANALYSES, FRESH WEIGHT BASIS. 1944

VARIETY OR STRAIN	THIAMINE	RIBOFLAVIN	ASCORBIC ACID	CAROTENE	DRY WEIGHT
	mcg./100 gm.	mcg./100 gm.	mg./100 gm.	mcg./100 gm.	%
Creole	304	97	37.0	408	21.5
Miracle	438	98	36.4	714	21.3
Morse Market	341	84	31.1	462	23.4
Perfection	338	96	36.4	390	21.8
Perfectah	341	97	34.8	406	22.5
Perfectah, Early	349	89	29.6	406	21.8
Walah	328	88	28.2	338	20.8
Wando	345	96	33.4	530	22.4
Willet's Wonder	305	117	45.7	522	24.4
1–12*	447	81	19.8	430	26.6
1–19*	188	89	21.3	471	27.0
903 (Giant Stride × Progress)	407	79	22.9	453	23.7
L.S.D. 5%	44	9	5.0	71	2.7
1%	62	12	7.1	100	3.8

* Uncertain pedigree.

Laxton × Phenomenon) cross are all much lower in carotene than those from the cross Little Marvel × World Record. Highly significant differences in carotene content were found among the four commercial varieties.

The second group of 12 later-maturing varieties (table V) were grown in randomized blocks in two field replicates, and two harvests were made from each replicate. All varieties from both replicates were harvested on the same day. The data were analyzed in a split plot design with replicates and varieties constituting the first split and with harvests and harvests × varieties the second split. All F values for replicates were non-significant.

The F values were significant at the 1% level for varieties for all vitamins, and for harvests for all vitamins except riboflavin. Table V gives the average values on the fresh weight basis. When the data from both groups of varieties were analyzed on the dry weight basis the F values were also significant for varieties for all vitamins.

Among the commercial varieties in this group Miracle stands out as the highest in both thiamine and carotene content. The strains 1–12 and 903
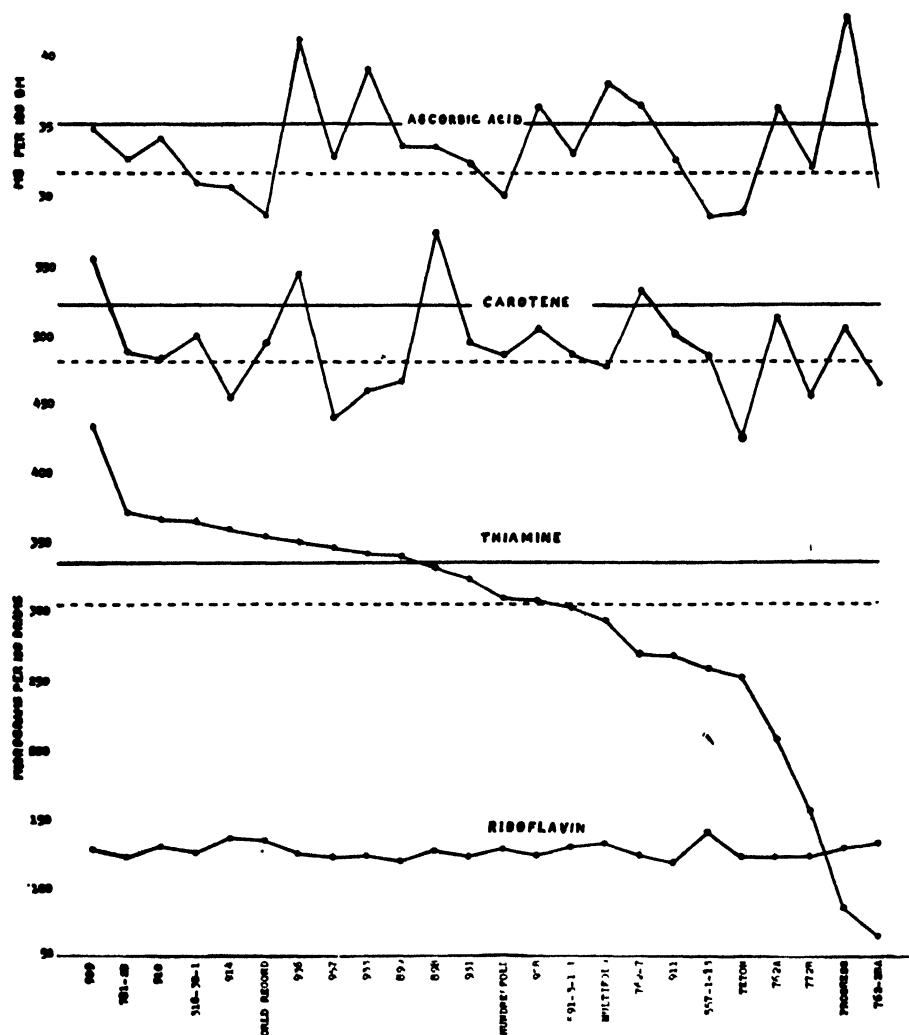
FIG. 1. Vitamin content of 24 varieties or strains of peas, 1945 experiment.

PEDIGREE OF STRAIN NUMBERS

CROSS	STRAIN NUMBERS
Little Marvel × (Thomas Laxton × Phenomenon)	909, 910, 911, and 781–2B
Progress × Kent Alderman	591–3–1–1, 931, 933, and 936
Progress × Giant Stride	762A, 762–2BA, 762–7, 898, 899, and 957
Progress × World Record	557–1–13, and 772B
Giant Stride × World Record	958
Giant Stride × Sutton's Excelsior	914
Phenomenon × Thomas Laxton	318–3B–1

The varieties having values above the solid horizontal lines are statistically significantly higher than those values appearing below the dotted lines.

were also high in thiamine content, while most of the other varieties have more of a medium range of values with the exception of the very low value

for strain 1–19. Willet's Wonder contained significantly more ascorbic acid and riboflavin than any other variety. This is consistent with its performance in 1943, when it also ranked the highest among the commercial varieties in these two vitamins.

In 1945 a simple lattice containing 25 varieties or strains and four replicates was used to give information on vitamin content as well as yield data. The individual varieties were harvested in April as they matured. All replicates of a variety were harvested on the same day. There was some difference in spread of the harvesting period for the several varieties due to slight differences in maturation rate. However, all harvests were completed within 12 days. The data were analyzed as randomized blocks except in the case of thiamine where the simple lattice design gave a sufficient gain in precision to warrant the use of values adjusted for block effect. One strain failed to produce enough to complete all analyses so it was omitted from the results presented in figure 1.

The varieties in figure 1 are arranged in a descending order according to their thiamine content. The division of the plotted values into three groups each for thiamine, for carotene, and for ascorbic acid content shows that a considerable number of varieties are significantly higher than others. If these same values are plotted on a dry weight basis for thiamine content several varieties change their relative ranking within groups, but there is only one exchange of varieties between groups and that occurs between the highest and intermediate.

The three strains 909, 781–2B, and 910, all derived from a cross involving Little Marvel × (Thomas Laxton × Phenomenon) ranked highest in thiamine content. These same strains were among the higher-ranking strains in an earlier experiment, table IV.

The strain 914 from the Giant Stride × Sutton's Excelsior cross ranked very high in thiamine content and quite low in ascorbic acid, just as it did in the 1943 survey data. It also gave quite a low carotene value in 1945.

Strains 936, 933, and 931 from the Progress × Kent Alderman cross are of interest. All three were medium to moderately high in thiamine content and the first two were definitely high in ascorbic acid content. Strain 936 appears to have combined in it the factors for relatively high thiamine, carotene, and ascorbic acid content along with an average amount of riboflavin. This strain is of particular interest in a program of breeding for higher vitamin content in peas.

Discussion

Statistically significant varietal differences were found in thiamine, riboflavin, ascorbic acid, and carotene content of peas over a period of several years. It is of interest to compare the nutritional significances of these differences. Using the data from the 1945 experiment and basing the daily allowances on those recommended by the National Research Council (1945), 100 gm. of the fresh, uncooked peas from the lowest-ranking varieties would

supply 4½% to 5% of the thiamine, 6% to 7% of riboflavin, about 14% of the carotene, and 37% to 40% of the ascorbic acid of an adult's daily requirement. In contrast, 100 gm. of the higher-ranking varieties would supply from 30% to 35% of the thiamine, 7% to 8½% of the riboflavin, about 19% of the carotene, and from 56% to 60% of the ascorbic acid. The proportions of the daily requirements actually supplied by these varieties when cooked will depend on losses in cooking. With the best available methods losses can be negligible, but under less favorable cooking conditions they may run as high as 30% of the thiamine and 60% or more of the ascorbic acid. It is readily seen that, from the nutritional standpoint, the consider-
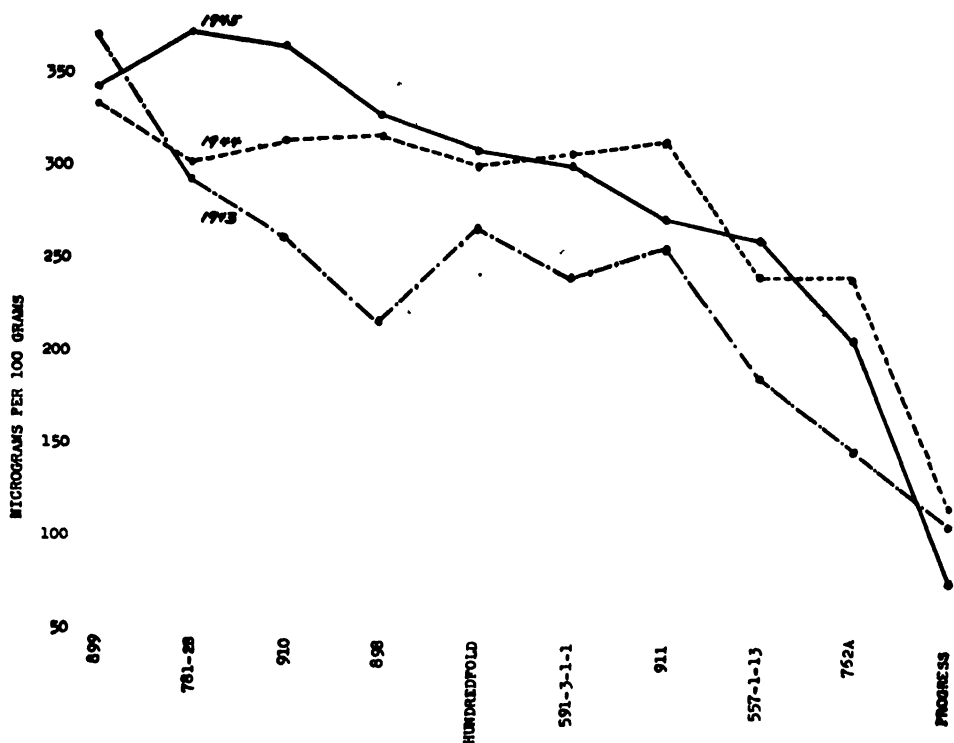
FIG. 2. Thiamine content of 10 varieties or strains of peas for a three-year period.

able variety variation in thiamine content is much more important than the relatively moderate variations in the other vitamins.

It should be pointed out that the varieties or strains represented in the 1945 experiment were all medium- or large-seeded types. If some of the small-seeded varieties such as Willet's Wonder could have been included in this study the nutritional significance of the varietal variation for ascorbic acid and riboflavin content might have been increased considerably. The difference in the time required for varieties of widely differing types to mature makes it difficult to compare any large number of varieties unless they are planted at different dates so as to bring them to the harvesting stage at approximately the same time. The early experiments in this study emphasized that the varieties being compared must be harvested over a relatively short period of time.

Ascorbic acid is the vitamin most easily lost or destroyed during cooking and preparation of the peas for serving. While the ascorbic acid content shows considerable varietal variation, the significance of this from a nutritional standpoint is thus much reduced. However, peas are generally considered a fair source of vitamin C.

The relative ranking of a few of the varieties over a period of years indicates that some varieties are quite consistent in their response insofar as thiamine content is concerned (fig. 2). Progress definitely ranked below any of the other varieties or strains in this group in each of the three years, and the breeding lines 899, 781–2B, and 910 were always high in thiamine content. Table I shows the ascorbic acid content of a number of varieties or strains over a period of two or more years. Although uniformity for ascorbic acid content from year to year is much less than for thiamine, some trends can be observed. If the eight varieties or strains for which three years' data are given are considered it will be noted that strain 762A ranked either highest or second highest in all three years. Likewise strain 781–2B was found to be near the bottom in ascorbic acid content over the three-year period. Among the strains for which only two years' data are given, 902 ranked the highest and the small-seeded variety Alah was second. Strain 902 also showed a relatively high thiamine content in 1944.

Correlation studies were made on the 1944 and 1945 data in an attempt to determine if any relationship existed between the quantities of each of the four vitamins present. In all experiments a negative correlation coefficient was obtained for ascorbic acid and thiamine content on the fresh weight basis, but the coefficient never approached a significance level in these studies.

There is a significant but not very important positive correlation between ascorbic acid and carotene content on the fresh weight basis. Correlation coefficients of 0.293 and 0.265 (significant at the five % level) were obtained for ascorbic acid and carotene content in the 1944 and 1945 data, respectively. Correlations involving all other vitamins were erratic from experiment to experiment and from year to year and very few of the coefficients approached a significance level.

In considering these strains as possible horticultural varieties it is of some importance to note their response in cold-hardiness tests. WADE (11) has shown that the strains 910, 898, and 899 included in this vitamin study were better than or equal to Progress in frost tolerance.

Summary

A number of varieties and breeding strains of garden peas have been analyzed for thiamine, riboflavin, ascorbic acid, and carotene content over a period of several years. Highly significant varietal differences have been found for all four vitamins.

The seed size, or stage of maturity, of the peas was found to have a significant effect on the vitamin content. On the fresh weight basis ascorbic acid decreased and thiamine progressively increased with increase in the

size of peas. On the dry weight basis the ascorbic acid and the riboflavin content decreased rapidly and thiamine remained nearly constant as the peas became more mature.

Correlation studies showed a slight significant positive correlation between ascorbic acid and carotene content. No significant relationship was found between the other vitamins.

The differences in thiamine content among pea varieties and breeding lines are large enough to be of real importance in an ordinary diet. Since thiamine content is found to be relatively consistent from year to year among varieties, it clearly merits attention in any breeding work designed to improve the nutritive value of peas.

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THE EFFECT OF AIR SUPPLY ON APPARENT PHOTOSYNTHESIS

JOHN P. DECKER

It has been recognized for a long time that the rate of absorption of CO_2 by a green plant shoot or leaf enclosed in a vessel is affected by the rate at which air is supplied. Much attention has been given to the problem of maintaining a "normal" air supply; that is, a CO_2 supply comparable to that found under natural conditions. The early literature was reviewed and discussed by HEINICKE and HOFFMAN (5), who presented results showing that the rate of photosynthesis was below normal when the air supply was less than approximately two liters per hour per square centimeter of leaf area. VERDUIN and LOOMIS (9) presented results from which they concluded that photosynthesis of corn leaves "was affected surprisingly little" by 70% depletion of the CO_2 within the leaf chambers.

BROWN and ESCOMBE (1) presented results which they interpreted as indicating an approximate proportionality between the rate of apparent photosynthesis and the mean CO_2 concentration. They pointed out that "in all cases where the illumination of the leaf was good, although the amount of intake of CO_2 into the leaf was approximately proportional to the increased partial pressure, the photosynthetic work was always somewhat in excess of what might be expected from the increased amount of CO_2." They were obviously expecting strict proportionality and had overlooked some of the following facts. Proportionality implies a linear relationship. General linear relationship is described by the equation $y = a + bx$; but strict proportionality exists only when $a = 0$ and $y = bx$. Apparent photosynthesis becomes zero at a measurable CO_2 concentration (the compensation point); therefore $a \neq 0$, and strict proportionality cannot exist.

The data of BROWN and ESCOMBE actually contain very little information concerning the real relationship between photosynthesis and CO_2 concentration. They made only two observations on each plant, one at high CO_2 concentration and one at low concentration. Thus only two points were established for each plant, and a linear function can be derived from any two points whether established experimentally or selected at random. Further, a variety of functions other than linear can be fitted to any two points, for example, $y = ax^n$, $y = a + 1/x$.

Data presented by DENEKE (4) indicated that the rate of photosynthesis increased with the velocity of air over the leaves and apparently approached a maximum limit at an air velocity of approximately 100 meters per minute. He circulated air over a plant in a closed system and measured the time required for a certain reduction of CO_2 content. One set of data, his figure 8, is reproduced here as figure 1. This and similar curves suggested to
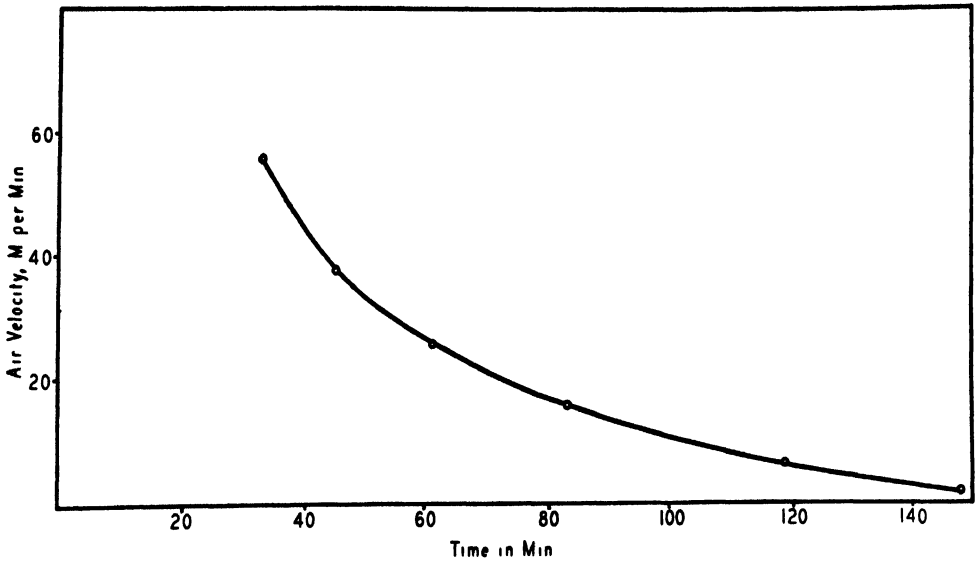
FIG. 1. Effect of air velocity on time required for a constant amount of apparent photosynthesis. Redrawn from DENEKE (4).

DENEKE that increasing velocity above 100 meters per minute would probably result in no further increase in the photosynthetic rate. However, it should be noted that rate is the reciprocal of time; that is, $R = K/T$ where R
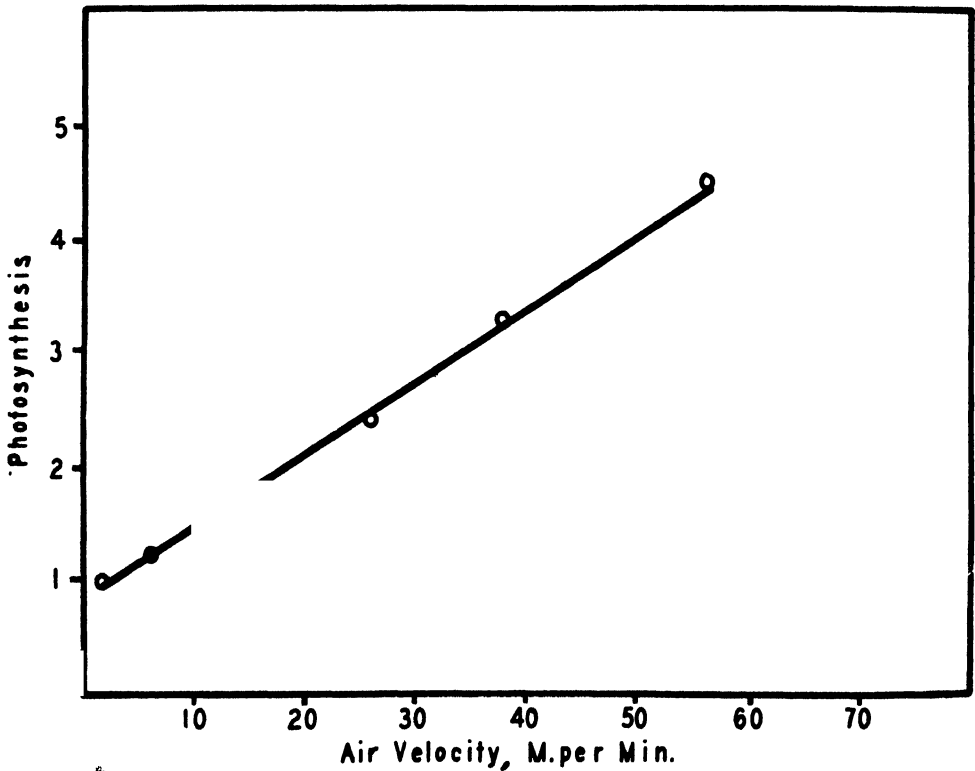
FIG. 2. Effect of air velocity on apparent photosynthesis. Recalculated from data of DENEKE (4).

is the rate of photosynthesis, and T is the time required for the absorption of a constant amount of CO_2, K. When photosynthetic rates were calculated from DENEKE's data they were found to describe a nearly straight line in relation to air velocity as shown in figure 2. DENEKE plotted T, the reciprocal of R, and the resulting curve was hyperbolic, of course. What he has interpreted as the flattening of the curve at high velocity is merely the normal shape of a hyperbolic curve.

HEINICKE and HOFFMAN (5) presented results which they interpreted as showing that the rate of photosynthesis declined more rapidly than the mean CO_2 concentration at very low rates of air supply. This implies a curvilinear relationship between rate of photosynthesis and mean CO_2 concentration. Values for mean CO_2 concentration were calculated from the data in their table 4 using the method they described, and photosynthesis was plotted over
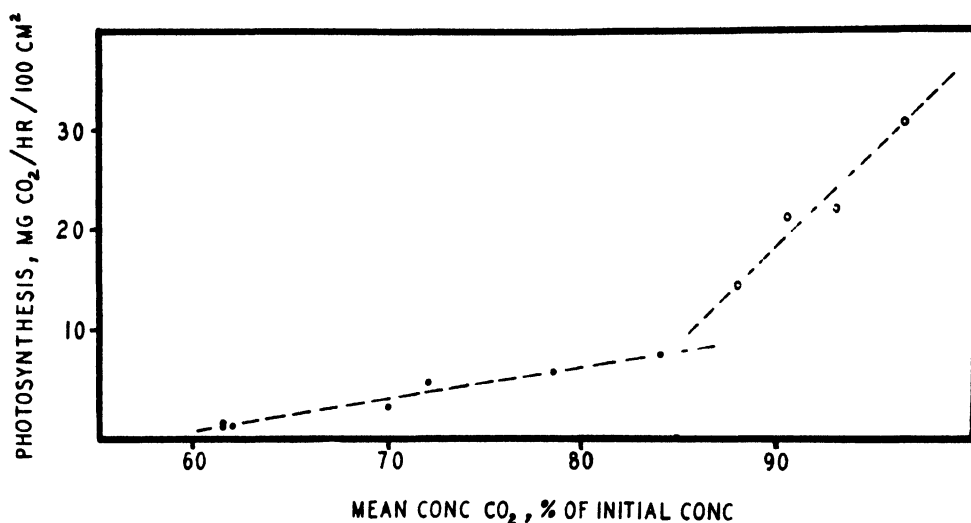
FIG. 3. Effect of CO_2 concentration on apparent photosynthesis. Data of HEINICKE and HOFFMAN (5).

mean CO_2 concentration as shown in figure 3. When the whole array is considered, a non-linear curve is suggested. It appears possible, however, that the array is composed of two sets of data describing two straight lines of different slope. It should be noted further that the four upper values were observations on single leaves and the remainder were composite measurements on many leaves. The inference of curvilinear relationship from these data is not justified.

HEINICKE and HOFFMAN stated also that the rate of photosynthesis fell rapidly when the CO_2 content was depleted more than 20%. This implies a curvilinear relationship between the rate of photosynthesis and the degree of depletion. When the data contained in their table 4 were plotted it was found, as before, that although the whole array suggested a non-linear curve, the data obviously could be separated into two groups. DECKER's (3) conclusion concerning the non-linear effect of CO_2 depletion on the photosynthetic rate of pine was based on a misinterpretation of preliminary results.

The experimentation reported here was undertaken in an attempt to clarify partially the effect of air supply on the rate of photosynthesis of the entire shoot of a tree seedling enclosed in a chamber.

Methods

The apparatus used in measuring photosynthesis was as originally described (3) except that CO_2 analysis units of the type used by WAUGH (10) were substituted. A diagram of the apparatus is shown in figure 4. The entire top of the plant was enclosed in a transparent cylindric chamber and was illuminated from above by a battery of electric lights. Air was drawn through the chamber at a known rate, and the difference between the amounts of CO_2 entering and leaving the chamber was taken as a measure of apparent photosynthesis. A small radial-flow fan (2) circulated the air rapidly within the chamber. A simple test with smoke showed that the turbulence caused by the fan was very much greater than that caused by the flow of air from inlet to outlet. It is presumed, therefore, that the rate of air movement within the chamber and over the leaves was essentially constant at the different rates of air supply. Air temperature in the shoot chambers was maintained at $25° \pm 1°$ C.

The arrangement of the CO_2 analysis units for direct sampling was similar to that described by WILSON (12). For simplicity only one of the units is shown in figure 4. Another was connected to the blank sampling tube (8) and a third to the other shoot chamber (7). The three units were arranged in a suitable thermostatic water bath. The sample was metered (14) through the absorption tower (15) which was charged with 50 ml. of 0.0055 N NaOH solution. The change in concentration of the NaOH was measured by the change in electrical resistance of the solution. The operating routine was as follows. The tower was charged with fresh solution and was stirred for five minutes by passing the CO_2-free air stream through it. The air stream was made CO_2-free by passing it through soda-lime (17). The tower was then by-passed and the initial resistance reading was made. The tower was again switched into the air stream and the soda-lime tube was by-passed for exactly fifteen minutes. The CO_2-free stream was then allowed to pass through the system for an additional three minutes to sweep the remaining CO_2 into the tower, the tower was by-passed, and the final resistance reading was made. At a CO_2 concentration of 0.55 mg./l. and an air flow of 0.55 l./min. a run of 15 minutes gave a resistance change of approximately 100 ohms. An Industrial Instruments Model RC–1B bridge was used, and with a decade variable resistance coupled in series it was possible to make readings to the nearest 0.5 ohm with considerable ease and speed. Replicated tests with the three units sampling a common source of air revealed an average discrepancy between units of approximately 1%.

The sampling unit drew air from the shoot chamber at a constant rate of 0.55 l./min. The total flow through the chamber was controlled by means of another air line and flowmeter (18) which was calibrated over the range 0.5
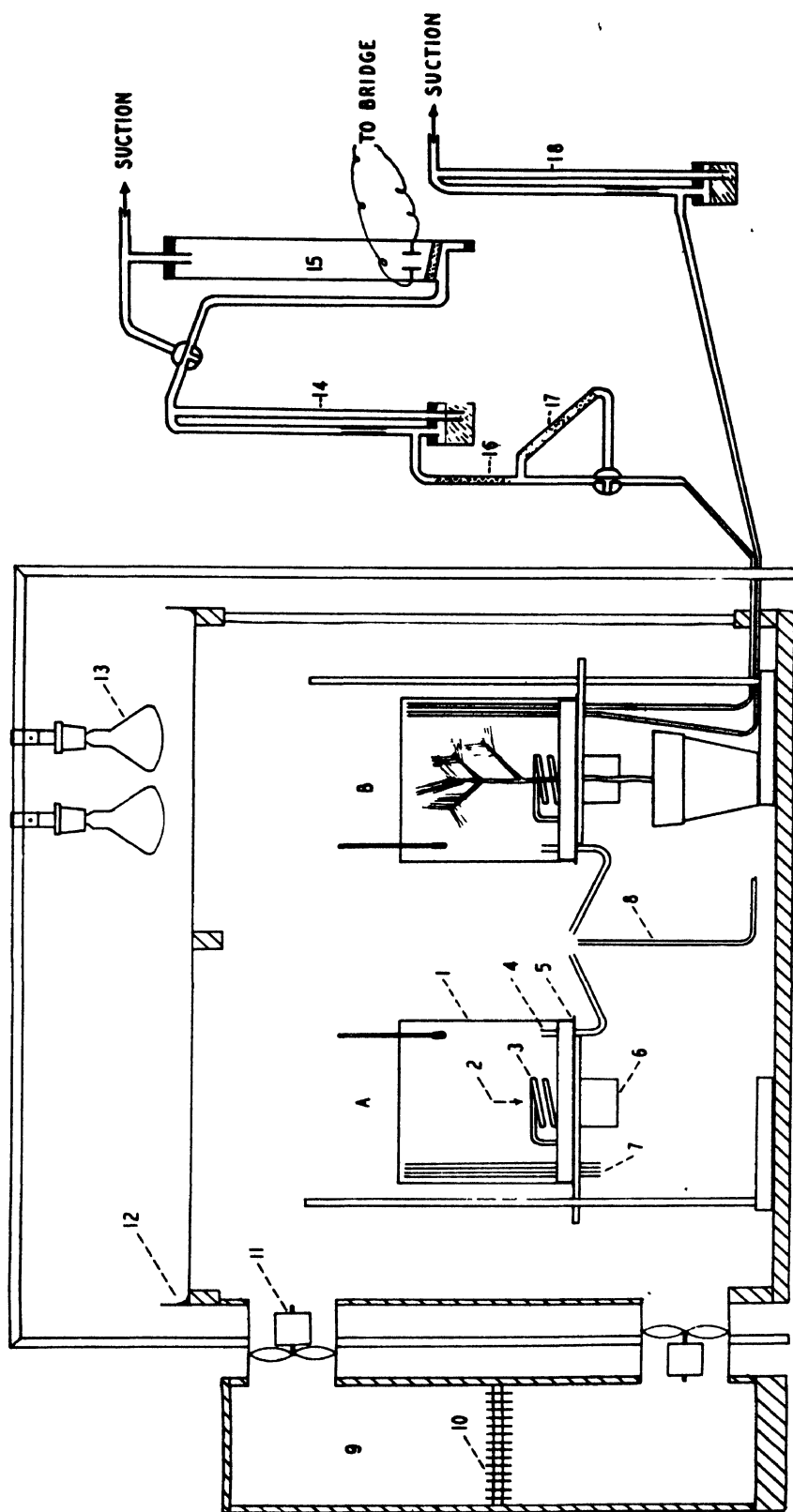
FIG. 4. Apparatus used to measure apparent photosynthesis of entire shoots under controlled conditions. Letters and numerals refer to descriptions in the text.

to 10.0 l./min. The desired rate of supply was established 20–25 minutes
before a measurement was begun. Preliminary tests made at the lowest rate
of air supply showed no difference between measurements begun 20–25
minutes after a change in rate of supply and those begun 45–50 minutes after
the change.

Ten potted loblolly pine (*Pinus taeda* L.) seedlings which had completed
the initial growth of their second growing season were used. Throughout the
season they had been exposed to normal light in a greenhouse. The experi-
ment was arranged as a 5×5 Latin square in which columns represented
hours of the day, rows represented pairs of plants and days, and the five rates
of flow were distributed within the square. To allow separation of any differ-
ential effect of light intensity, one plant of each pair was exposed to a light
intensity of 10,000 foot candles and the other to 2,200 fc. throughout the
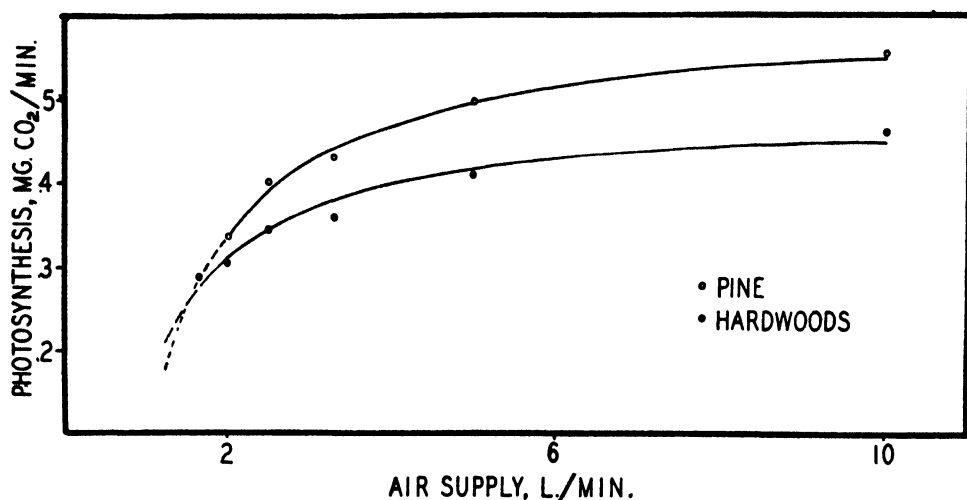
FIG. 5. Effect of air supply on apparent photosynthesis of entire shoots of tree seed-
lings. Data of table II.

series of flows. The Latin square design was chosen because it would reduce
the chance of having the decline of the CO_2 content of air from out-of-doors
through the day confounded with the effect of air supply. All plants were
exposed to all rates of supply and thus the amount of tissue involved was a
constant whose effect did not appear in differences between rates of supply.

The results of HEINICKE and HOFFMAN indicated that the rate of photo-
synthesis could probably be expected to vary hyperbolically with the rate of
air supply and therefore linearly with the reciprocal of air supply. The
rates of supply which were used (2.0, 2.5, 3.3, 5.0, 10.0 liters per minute) were
chosen because the reciprocals were convenient values.

A second series was run using six dogwood (*Cornus florida* L.) seedlings
and six tulip poplar (*Liriodendron tulipifera* L.) seedlings. These plants
were in their third growing season and were kept in the greenhouse near the
pine seedlings. A design similar to the previous one was used, with a 6×6
square, one plant of each species in a pair, and a sixth rate of supply of 1.67
l./min. Only one light intensity, 10,000 fc., was used in this series.

TABLE I

APPARENT PHOTOSYNTHESIS OF ENTIRE SHOOTS AT DIFFERENT RATES OF AIR SUPPLY.
MG. CO_2/MIN./SHOOT

SPECIES	LIGHT INTENSITY	AIR SUPPLY, L./MIN.					
		10	5	3.3	2.5	2.0	1.67
	fc.						
Loblolly pine*	10,000	.579	.531	.464	.441	.366	....
" " *	2,200	.530	.470	.401	.361	.309	....
Dogwood†	10,000	.414	.371	.318	.310	.282	.260
Tulip poplar†	10,000	.519	.455	.409	.383	.333	.318

* Values are means of 5.
† Values are means of 6.

Results

Results are summarized in tables I and II. Values for mean CO_2 concentration and degree of depletion were calculated from the mean initial and final concentrations within each air supply group.

There is an obvious difference between the values for the two light intensities with pine; however, an analysis of variance (table III) revealed no differential effect of air supply with light intensity, and the two sets of data were combined for all further considerations. Similarly, no differential effect of air supply with the two hardwood species was found, and the data for the two species were combined. The statistical significance of row and column effects indicates that the Latin square design gave a real increase in precision.

Regressions were fitted to the two sets of data using 1/R (where R is the rate of air supply) as the independent variable and rate of CO_2 absorption as the dependent variable. Both were found to be linear. The analyses of variance are given in table III. The linear curves became hyperbolic, of course, when transformed from the reciprocal scale to the normal scale as shown in figure 5.

TABLE II

APPARENT PHOTOSYNTHESIS, MEAN CO_2 CONCENTRATION AND DEGREE OF DEPLETION
OF CO_2 AT DIFFERENT RATES OF AIR SUPPLY

		AIR SUPPLY, L./MIN.					
		10	5	3.3	2.5	2.0	1.67
Pine	Photosynthesis (mg. CO_2/min.)	0.555	0.501	0.432	0.401	0.337	....
	Mean CO_2 conc. (mg./l.)	0.520	0.495	0.479	0.465	0.456	....
	% depletion	10.2	18.4	23.8	29.4	31.2	....
Hardwoods†	Photosynthesis	0.466	0.413	0.363	0.346	0.307	0.289
	Mean CO_2 conc.	0.508	0.494	0.473	0.463	0.453	0.448
	% depletion	8.8	15.1	20.9	26.3	29.3	31.9

* Values are means of 10.
† Values are means of 12.

TABLE III
ANALYSIS OF VARIANCE

PINE			HARDWOODS		
SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE
Rows	4	.077205	Rows	5	.002735
Columns	4	.001496	Columns	5	.022684
Flows	4	.072026	Flows	5	.052861
Error	12	.002782	Error	20	.003940
Total	24		Total	35	
Light	1	.047678	Species	1	.106650
Light-Flow	4	.000305	Species-Flow	5	.001221
Error	20	.009930	Error	30	.002864
Total	49		Total	71	
Regression*	1	.028569	Regression*	1	.021260
Residuals	3	.000081	Residuals	4	.000195
Total	4		Total	5	
$b = -.54 \pm .09\dagger$			$b = -.35 \pm .09\dagger$		

* Regression tables are based on means of 10 and 12 observations respectively.
† Standard error of b multiplied by 5% t.

The curves of apparent photosynthetic rate plotted over the degree of depletion (not shown) appeared to be linear with essentially the same dispersion of points shown in figure 6.

Discussion

These data are in general agreement with the conclusion of HEINICKE and HOFFMAN that the effect of air supply on CO_2 absorption is curvilinear
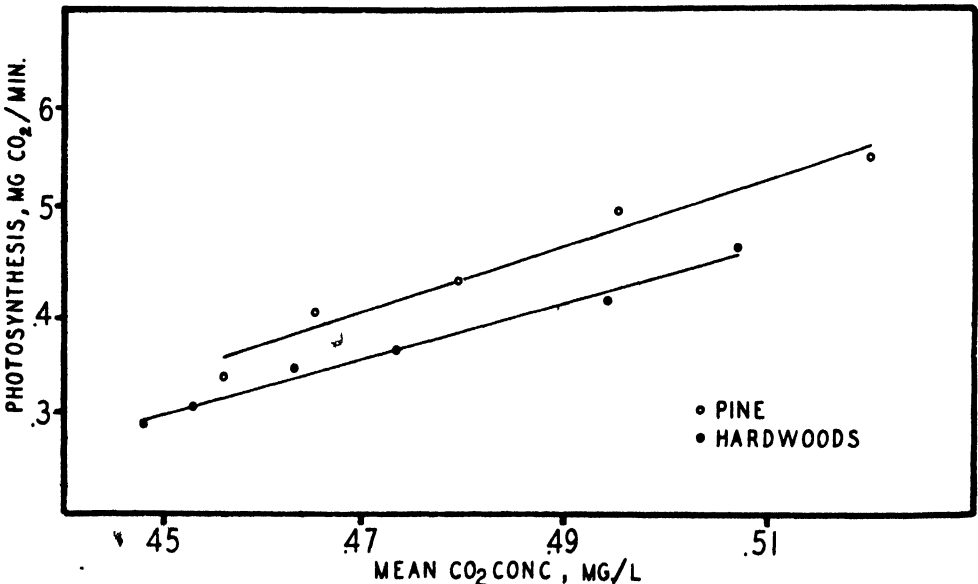
FIG. 6. Effect of CO_2 concentration on apparent photosynthesis of entire shoots of tree seedlings. Data of table II.

over the known range. There is apparent disagreement with DENEKE's data, which showed a linear relationship; however, the technique used by DENEKE differed from that used in the present study. DENEKE measured the time required for a standard depletion of the CO_2 content of a fixed volume of air in a closed system, thus the mean CO_2 content was constant for all air velocities. It appears that he measured directly the simple effect of air velocity over the leaf. In the present study the air velocity was held essentially constant and the mean CO_2 content was varied.

Considerable attention has been given in past studies of photosynthesis to the problem of maintaining a "normal" or at least known CO_2 supply. A frequent practice has been to maintain an air supply such that the CO_2 content was reduced not more than 15–20%. This practice is subject to question because it has been based on the conclusion of HEINICKE and HOFFMAN that the effect of depletion on the photosynthetic rate increases rapidly at depletions greater than 20%. There is another objection to the use of degree of depletion in attempting to evaluate or control the effect of CO_2 supply. Degree of depletion is a relative value and does not take into account the actual CO_2 pressure, thus 20% depletion when the initial concentration was 0.6 mg./l. is different from 20% depletion when the initial concentration was 0.4 mg./l. The mean CO_2 content of air as used by BROWN and ESCOMBE, that is, the arithmetic average of the affluent and effluent air, is probably a more useful value. The relationship between rate of photosynthesis and mean CO_2 concentration found in the present experimentation is shown in figure 6. It is proposed that for a given set of experimental plants a similar regression could be established, by means of which all further experimental measurements of photosynthesis could be corrected to a selected mean CO_2 concentration. The effect of CO_2 supply could thus be eliminated from comparisons between measurements. In such a regression proper account should be taken of the fact that the independent variable, x, is subject to considerable sampling error.

Apparently it is generally supposed that, because the volume percentage concentration of CO_2 in air is essentially constant at all altitudes, CO_2 is not one of the determinative factors in the altitudinal distribution of species. The writer has been unable to discover any direct evidence supporting this supposition. LUNDEGARDH (6) discussed CO_2 as an ecological factor but made no mention of it in relation to altitude. WEAVER and CLEMENTS (11) did not mention it. MEYER and ANDERSON (7) stated that because CO_2 concentration is nearly constant it seldom need be considered a variable in interpreting the developmental behavior of plants under natural conditions. However, the diffusion of CO_2 into a leaf is a function of CO_2 pressure rather than concentration, and the pressure of CO_2 in the atmosphere varies directly as the total atmospheric pressure. The normal pressure of atmospheric CO_2 is approximately 22.8 mm. Hg at sea level and 13.0 mm. Hg at 15,000 feet altitude. DAUBENMIRE (2) recognized that the partial pressure of CO_2 varies with atmospheric pressure and stated that its effect on plant growth is indirect.

From the relationship between photosynthesis and CO_2 concentration shown in figure 6 a similar relationship between photosynthesis and CO_2 pressure may be deduced, for CO_2 pressure is a direct function of CO_2 concentration at constant total pressure. These data indicate a possible differential response to CO_2 pressure of the photosynthetic mechanisms of species normally growing at the same altitude.

In general, the natural distribution of vegetation is determined by the differential responses of species to gradients in one or more environmental factors. It seems reasonable to expect, therefore, that some species characteristic of different altitudes may exhibit differential responses to CO_2 pressure which are related to distribution, and thus at times the gradient in CO_2 pressure might be one of the significant factors of the determinative complex.

Summary

The apparent photosynthetic rate of the entire shoots of tree seedlings was found to vary hyperbolically with the rate of air supply and linearly with mean CO_2 concentration over the range 0.52 to 0.45 mg./l. The possible importance of CO_2 pressure as a factor in altitudinal distribution of species is suggested.

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ARTIFICIAL PARTHENOCARPY IN *LYCOPERSICUM ESCULENTUM*; TISSUE DEVELOPMENT

GENEVIEVE N. KING

(WITH SEVENTEEN FIGURES)

Induction of parthenocarpy and a comparison of normal and parthenocarpic fruits was undertaken in order to determine the possible role of the embryo in the development of the fruit. There is at present a question as to whether the production of fruit is brought about as a result of growth substances from the pollen, the developing ovule, seeds, tissues of the ovary wall and placentae, or from some other region of the plant. These arguments have been reviewed by GUSTAFSON (**9, 10, 11, 12**), and it is believed that the data given below contribute further to the interpretation of this point.

Materials and methods

The Marglobe variety of *Lycopersicum esculentum* was grown under greenhouse conditions, with a temperature range from approximately 70°–85° F., and the experimental work was carried out with seventy selected plants. One group received the treatment necessary for the production of parthenocarpic fruit; the second group was kept as a control.

The seeds were planted October 6, and the seedlings were transplanted several times to secure a vigorous root system; they were kept under observation from that time until February 2, when the first treatment was applied.

Indolebutyric acid in lanolin was prepared in the manner reported by OLESON (**20**), and used in concentrations of 0.5% by weight. The flowers were emasculated, to prevent pollination, before the bud opened. Both the sepals and petals were removed with a minimum of trauma after the blossom opened in order to facilitate the application of the lanolin smear.

Applications were initially made in several ways: on the stigma, on a horizontal cut at the base of the style, or at a cut through the ovary. Parthenocarpic fruits most nearly resembling the normal fruits were produced by the treatment of the cut at the base of the style as described by GUSTAFSON (**9**). This method of treatment was used throughout the remainder of the experiment. Following the application of the lanolin-indolebutyric acid mixture, the flowers were bagged with cellophane envelopes to prevent any contamination by pollen, although it has been reported that the pollen becomes inactivated when in contact with lanolin mixtures containing some growth-promoting substance. Treatments were continued on the blossoms as they opened through February and March.

Beginning March 2, following the above treatment, flowers were cut below the abscission layer of the pedicel and collected at two-hour intervals for a period of twenty-four hours; further collections were made at 24-hour

intervals over a period of two weeks to obtain samples showing the changes as they took place following the application. Samples were also collected of comparable stages of the flower following pollination. Records were made of the general development of the treated and control plants as well as of the normal and parthenocarpic fruits. These records included the rate of fruit development following pollination and the treatment with lanolin smear, ultimate size and weight of the fruit, increase in size of the pedicel, and the premature abscission of flowers and fruits. ·

All tissue samples were preserved in formalin-acetic-alcohol and embedded in paraffin following dehydration with n-butyl alcohol. Sections were cut 10–12 microns in thickness and stained with safranin and fast green. Serial sections of important developmental stages were cut in both transverse and longitudinal planes to follow the sequence of events in the early development of normal and parthenocarpic fruits.

Data and discussion

The artificial induction of parthenocarpic fruit in the tomato by the use of a 0.5% by weight of indolebutyric acid in lanolin at the base of the style parallels the work of SCHROEDER (21) and HOWLETT (16). The development of the fruit was much more rapid following the treatment with the lanolin smear than as a result of natural pollination but the ultimate weight and size of the fruit were approximately the same. The parthenocarpic fruits produced were more solid, and the percentage of set of fruit was as great, or greater, than that of the normal fruit. Larger fruits were produced if the treatment followed partial pollination. The percentage of fruit-set was increased above that of the controls if repeated applications of the stimulant were made at short intervals. In agreement with the results obtained by HOWLETT (16, 17), parthenocarpic fruits were produced on the same plants with fruits producing seeds and their growth was not impaired if repeated indolebutyric acid treatments were given. Histological studies were made of the early stages of flowers and fruits of the normal and treated plants in order to compare their development.

The development of flowers and ovules has been described by COOPER (4, 5). The Marglobe tomato has five or six locules, usually six, and free central placentation (fig. 1). It is impossible to get an accurate idea of the developments within the embryo sac with sections 10–12 microns in thickness such as were used in this investigation. It is possible, however, to know the condition of the ovule in the flower at the time of pollination (fig. 2) and at the time of the application of the lanolin-indolebutyric acid mixture (fig. 4) and thus to follow the subsequent changes.

The tomato fruit consists of pericarp, ovules, and placental tissue. The greatest changes during fruit development take place in the ovules and the placentae. The development has been accurately described by HAYWARD (14) and general descriptions of parthenocarpic fruits have been given by several authors (5, 6, 7, 9, 10, 11, 12, 13, 16, 20, 21, 22). The development

of the placental tissue of the parthenocarpic fruits, however, has not been stressed. A critical study of the parthenocarpic sections showing placental tissue at the time of pollination (fig. 1), one day after treatment (fig. 3),
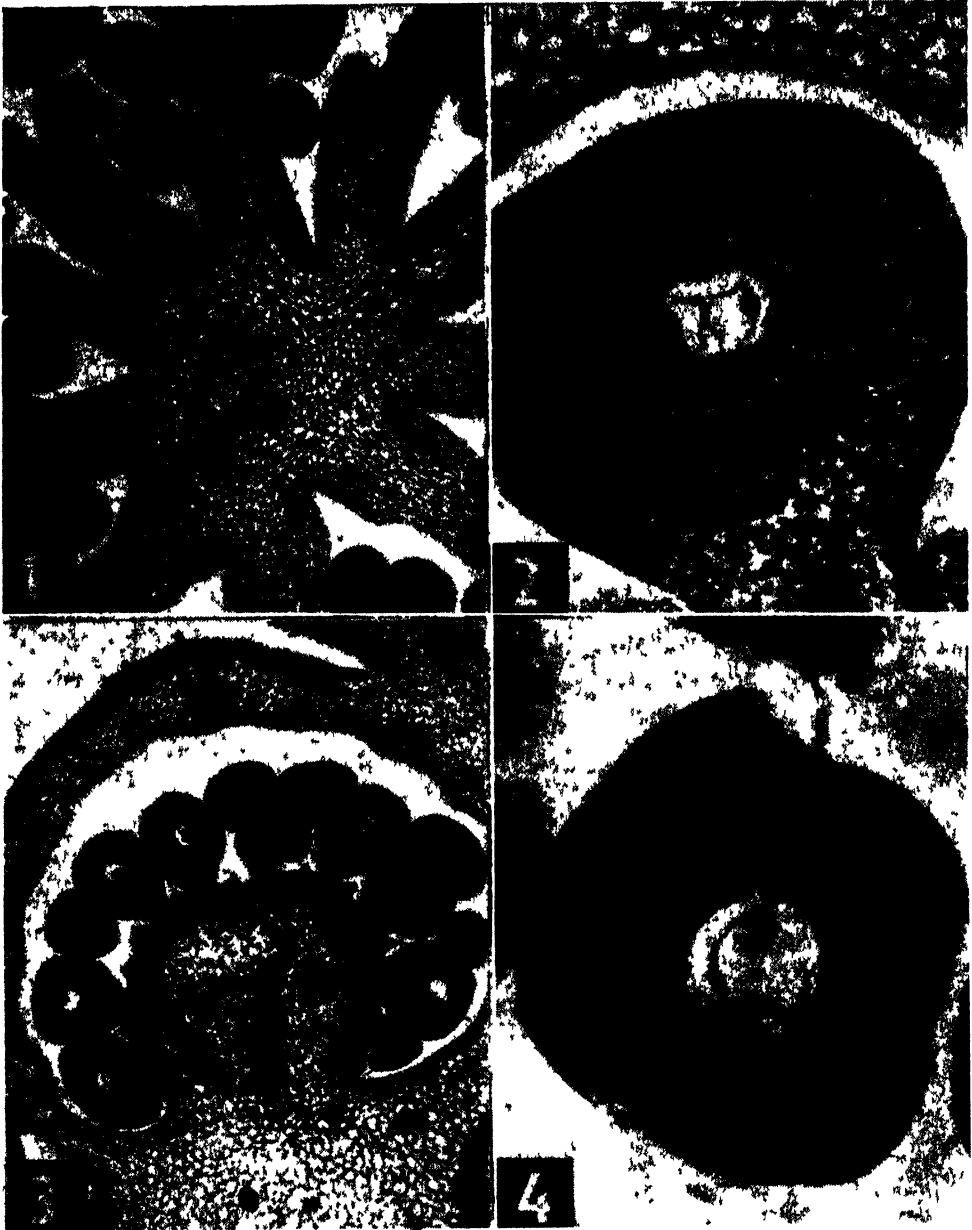
FIG. 1. Transverse section through the ovary of the flower showing free central placentation. FIG. 2. Section through the ovule at the time of pollination. FIG. 3. Longitudinal section through the ovary one day after treatment, showing placental tissue. FIG. 4. Section through the ovule one day after treatment.

and ten days after treatment (fig. 5), show the marked overgrowth of this tissue which in the mature fruit crushes the ovules against the ovary wall. This development is brought about by an increase in cell number and cell

size as stated by GARDNER and KRAUS (5). The general pattern of cell shape and cell size will vary among plants, due apparently to genetic influence (15). In fruits produced by the treatment following a horizontal cut through the young ovary (fig. 16), the growth of the placentae takes

FIG. 5. Longitudinal section through the ovary ten days after treatment, showing placental tissue. FIG. 6. Section through the ovule ten days after treatment, showing the collapsed embryo sac. FIG. 7. Longitudinal section through the pedicel, directly below the receptacle region of the flower approximately ten days after pollination. FIG. 8. Same as figure 7, showing details of vascular tissue.

the form of finger-like projections. The site of ovule attachment may be observed as scar tissue on the surface of these projections. The unfertilized ovule usually ceases its development early but if there is enlargement, it is due to the slight development of integument and nucellar tissue (5). The
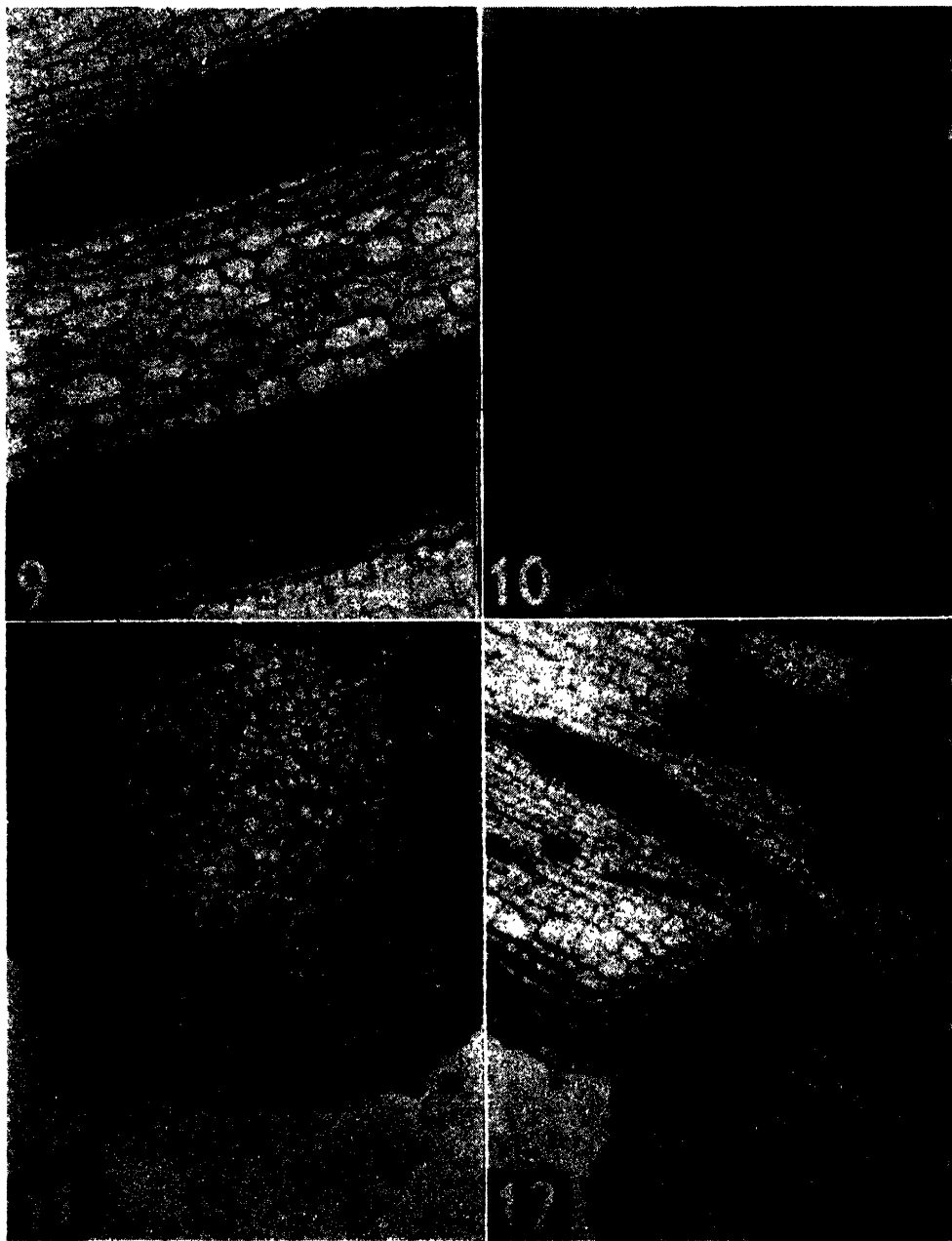
FIG. 9. Longitudinal section through the pedicel, directly below the receptacle region of the flower, approximately ten days after treatment. FIG. 10. The same as figure 9, showing details of vascular structure. FIG. 11. Transverse section through the abscission region of the pedicel, of the control showing the meristematic cells of the cortex. FIG. 12. Longitudinal section through the abscission region of the pedicel, of the control showing the meristematic cells of the cortex.

megagametophyte collapses a few days after the treatment with indole-
butyric acid as noted by BORTHWICK, HAMNER, and PARKER (2).

The influence of the lanolin-indolebutyric acid mixture extends through
the fruit into the pedicel and prevents abscission of flower and fruit. There
are many references in the literature on this point (2, 5, 6, 7, 8, 12, 16, 19).
Further data have been given by GARDNER and MARTH (6) who state that
flower buds could be stimulated to renew growth even after the pedicel had
begun to turn yellow, and that this increase of growth in the pedicel in the
Solanaceae was even more rapid than that following pollination. This
rapidity of effect would be expected in consideration of the rapid response
secured by ZIMMERMAN, HITCHCOCK, and WILCOXON (22) in their work with
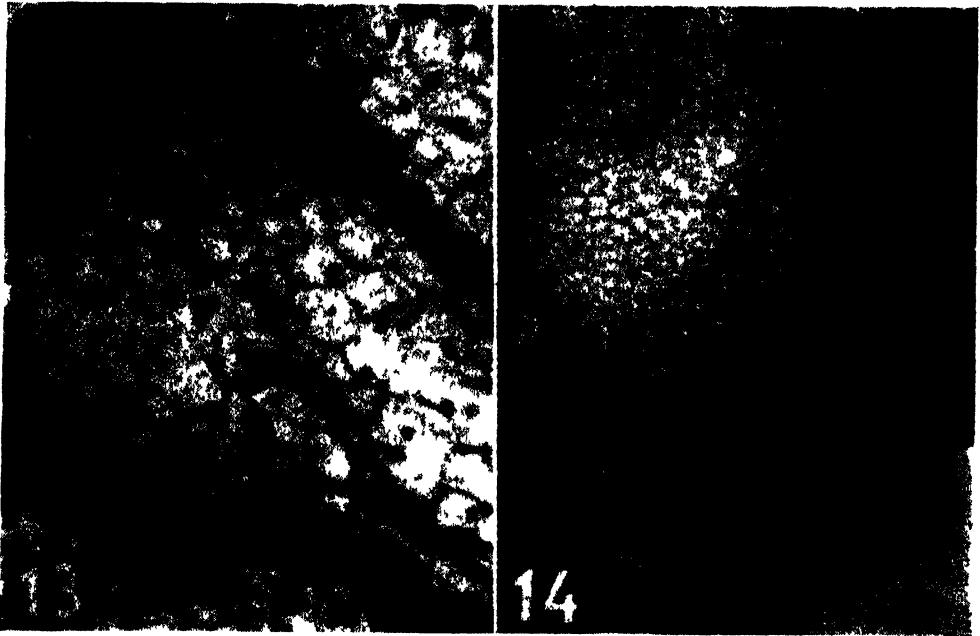
FIG. 13. The same as figure 12, showing details of vascular tissue. FIG. 14. Trans-
verse section of the abscission region of the pedicel, of the control showing the meristematic
cells of the pith.

the tomato. A comparison of the sections of the pedicel of the normal and
parthenocarpic fruit taken directly below the receptacle region (figs. 7, 8,
9, 10), show a distinct difference in the amount of vascular tissue. The
pedicel of the parthenocarpic fruit has more secondary tissue and larger
cells in the pith and cortex. These data also agree with the work of
KRAUS, BROWN, and HAMNER (18) as to the effect of growth-promoting sub-
stances on stem tissues. Contrary to the above results, GARDNER and KRAUS
(5) report that it is not possible to detect any direct outstanding differ-
ences in the vascular system of fruit or pedicel in comparing normal and
parthenocarpic fruit. The difference noted by the writer may be due to the
repeated applications of lanolin-indolebutyric acid mixture on the base of
the style. In the abscission region of the control the meristematic activity

is noticeable in both the cortex and pith regions and there is a separation or branching of vascular strands (figs. 11, 12, 13, 14). The difference in the pedicel of the normal and parthenocarpic fruits is not restricted to the internal anatomy but may be observed in the external appearance (figs. 15, 16, 17) as an increase in thickness over that of the normal fruit extending from the receptacle through the abscission region.

The theories of the causal mechanism of fruit development are reviewed by GUSTAFSON (9, 10, 11, 12). From the experimental work discussed, it is evident that the ovary wall is not important to any great extent in the production of growth-promoting substances which result in the development of fruit. When ovules are removed, fruit will not develop unless the cavity which is left is filled with a mixture containing a growth-promoting substance. The question arises as to whether the placentae, ovules, or embryo are responsible for the development of the fruit. In parthenocarpic fruits
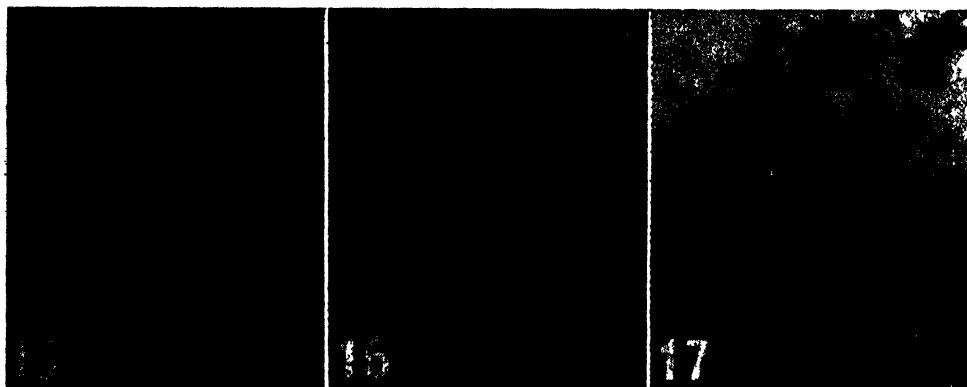
FIG. 15. Parthenocarpic fruits produced by the application of lanolin-indolebutyric acid smears, applied at the base of the style (×2). FIG. 16. Parthenocarpic fruits produced by the application of lanolin-indolebutyric acid smears, applied on a horizontal cut through the ovary (×0.5). FIG. 17. Marglobe tomatoes as developed from pollination followed by fertilization (×0.33).

which are seedless and in which ovules do not develop, it is evident that the development could not have been brought about by the ovules or the embryo sac. In the work of TUKEY (22), the other possible alternative is discussed and his data show that the abortion of the embryo may cause the early ripening of the fruit. Parthenocarpic tomato fruits may appear in the field at certain seasons of the year and it is believed that such cases may be explained on the basis that pollination has occurred and that the pollen tube with its contents in the style is responsible for the development of the fruit even though fertilization has not occurred (13). GUSTAFSON (9) states that the high auxin content in the flower bud stage is responsible for parthenocarpy. It is evident from the experimental work discussed by the same author that there is an increasing gradient in auxin concentration from the ovary wall to the developing seed. If the region of greatest concentration in the developing fruit is eliminated, it is logical to assume that

the region next in concentration would take over the control of mobilization of materials into the fruit. Such regions in order of decreasing concentration would be ovules, placentae, and ovary wall. The production of parthenocarpic fruit when the ovules fail to continue their development, as described in this paper, shows that the placenta itself may be stimulated to fruit development without the influence of the developing ovule.

The production of induced parthenocarpic fruits in which the ovules fail to continue their development beyond the stage reached at flowering would lend support to the theory that the ovule is not essential for fruit production. Other tissues of the fruit itself, such as the placentae, may be either the source of the stimulus for fruit development or the loci at which that stimulus exerts its effect. It is possible that the auxin gradient in fruit is determined by the tissue pattern established by the flower or floral primordia.

It is interesting to note in connection with the abscission layer that the vascular strands in that area are smaller; in many cases only a single row of tracheal elements. The tracheids retain their nuclei and cytoplasm over a much longer period than previously believed. Such conditions show a distinct adaptation of the structures of the abscission layer to its function which once again emphasizes the dynamic nature of plant tissues.

Summary

1. The parthenocarpic fruits produced by the application of 0.5% indolebutyric acid in lanolin were seedless, solid, juicy, and as large as the normal fruit. The yield was as high or higher than the normal fruit and when produced on the same plant with the normal fruit their development was not affected.

2. The ovules of the parthenocarpic fruit failed to continue their development to any degree after the stage at which the flowers were treated.

3. The embryo sac of the partially developed ovules of the treated flowers collapsed and the ovules were crushed against the ovary wall by the overgrowth of the placental tissue.

4. There was an excess tissue development in the pedicel of the parthenocarpic fruit as shown by cross sections, and this increase in the thickness of the pedicel is due to an increased amount of secondary tissue.

5. Parthenocarpic fruits produced on the same plant with normal fruits were as large as those produced on separate plants.

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THE POROMETER METHOD FOR THE CONTINUOUS ESTIMA-
TION OF DIMENSIONS OF STOMATES

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(WITH FOUR FIGURES)

In studies concerning the movement of gases and vapors into and out of the leaf, it is frequently necessary that the dimensions of the stomates be determined. Such information may be more useful if it consists of a continuous record of the stomatal aperture rather than a series of periodic measurements. Two methods are generally used for the procurement of such continuous data; direct microscopic measurement and the porometer.

Direct microscopic measurement of the dimensions of the stomates has been considered the most accurate method by many workers. There are, however, several difficulties which make accurate measurements by the microscope difficult. For certain leaves, under some conditions, accurate measurements can be made only with extreme difficulty. For other leaves, the microscope cannot be used at all. The method is not well adapted for continuous observation, because of the strain imposed upon the worker and because of undesired changes induced through mechanical manipulation and the use of light. Also, since only a few stomates can be measured at one time, the question arises as to whether they constitute a representative sample or reflect correctly the average stomatal aperture. However, when employed for the determination of the dimensions of the stomates at a particular instant, a high degree of accuracy may be achieved.

The porometer is a device used to measure changes in the rate of flow of air through the leaf. A cup or tube is attached to the leaf and the pressure of the air within the cup is either raised or lowered with respect to normal air pressure. It is assumed that the changes in the rate of air flow are directly proportional to the changes in the dimensions of the stomates.

The porometer was first proposed by Dutrochet in 1832, according to DARWIN and PERTZ (3). In 1876, Muller constructed an apparatus employing this principle which proved too complicated and cumbersome for general use and acceptance. The modern design for the porometer was developed by DARWIN and PERTZ (3). Improvements were made on this type of porometer by KNIGHT (7), and by LAIDLAW and KNIGHT (9) who devised a constant flow aspirator and made the process self-recording. Other porometers were devised by BALLS (2), and by JONES (6). A porometer which measures changes in the rate of flow of air through the leaves in terms of pressure was devised by GREGORY and PEARSE (4). This method uses small rates of air flow and low pressures. It will be described in detail in another section.

Numerous criticisms have been leveled at this method, many of which are no longer valid in the light of present information. LOFTFIELD (10) considered that until a "comparison has been made between a series of direct observations upon the conditions of the stomata and porometer readings made at the same time—its reliability is questionable."

ASHBY (1) compared readings of a porometer with simultaneous measurements from epidermal strips. He concluded that with large apertures the two methods are essentially measuring the same thing but that with small apertures the porometer method is more than ten times as sensitive. He also found the error of replication of the porometer to be less than 3% while that of the strip method was over 20%. MASKELL (11) compared the porometer rate with the area of the stomates determined from impressions. Although he made only a small number of comparisons, his results indicated a linear relation between porometer time and the area of the stomates.

In a study of the effect of certain gases on permeability of the leaves, PAWLENKA (13) made a great many simultaneous measurements of the stomates using the porometer and the strip method which were reinforced by direct microscopic observations. Although not intended as a check upon the porometer, it affords the most extensive comparative investigation yet reported. Invariably, there was a direct relation between the porometer time and the area of the stomates as determined by the strip method which was constantly checked by direct microscopic observation. HARTSUIJKER (5) after a critical study of the most important methods to determine the dimensions of the stomates, concluded that there was a direct relation between the porometer time and the area of the stomates as measured by direct microscopic observation. He considered that the porometer was an extremely useful device for the quantitative investigation of the changes in the dimensions of the stomates.

The rate of the passage of air through the leaf is also influenced by the resistance of the cells of the mesophyll. If the intercellular volume were to change appreciably during the course of an experiment, the porometer rate would be a reflection of this change as well as of changes in the dimensions of the stomates. NIUS (12) investigated this question and concluded that such changes actually occurred. If Nius' conclusions are correct, a serious and uncontrollable error would affect the accuracy of the porometer method. This question was reinvestigated by HARTSUIJKER (5) who concluded that Nius' methods contained so many errors that his results were not valid. By the use of improved methods of a known small error, he showed that the effect of changes in the intercellular volume is extremely small. The writer has repeated these experiments of Hartsuijker and confirmed his results

KNIGHT (8) showed that some stomates tend to close when air is drawn through them continuously. This difficulty is associated with the use of high pressures and large displacement of volume. If the pressure used is small and the volume of air drawn through the leaf reduced to a minimum,

stomatal action appears to be unaffected. The writer has obtained records from plants growing under controlled conditions in which the stomates, as measured by the porometer, were continuously and evenly open for more than twenty-four hours at a time. It would appear that the method itself, even though used continuously, does not induce closure.

The porometer method has several decided advantages when compared to other methods. All measurements are based on the action of a large number of stomates. For comparable results to be obtained by the direct methods, several thousands of stomates would need to be measured at each reading. Since the porometer reading is determined by the average aperture of the stomates, there is a small standard error of replication. The writer has tested this point repeatedly and has found the error to be within 3%. The small standard error implicit in this method permits accurate measurement of small changes in aperture. A further important advantage is that direct, continuous records of the movements of the stomates may be obtained under either natural or controlled conditions. The writer has obtained continuous records for periods of more than four months from plants growing out-of-doors.

The porometer method for determining the apertures of the stomates is apparently accurate, and suitable for continuous operation. It is felt that the objections which have been raised against this method, are either based on insufficient data or have been met by improvements in the method. It is considered that for most studies dealing with the effects of environmental factors upon the activities of the guard cells, the use of the porometer affords the possibility of accurate continuous quantitative measurement.

The resistance porometer

Since the porometer method has so many advantages, considerable attention was given to the development of a simple, reliable recording instrument. The resistance porometer, divised by GREGORY and PEARSE (4) is considered the most useful for precise continuous measurement of the aperture of the stomates under practical conditions. The apparatus which they used in recording the behavior of the porometer is both cumbersome and expensive and limits the usefulness of the porometer to the laboratory. A recorder was devised by the writer which is simple to operate and inexpensive to construct and has the additional advantage of requiring a very low pressure differential for its operation.

The method may be explained by reference to figure 1. A leaf, which when attached to a leaf cup behaves as a variable resistance, is connected through a fixed capillary resistance to a vacuum line. A tambour which is a flanged metal cup approx. 1″ in diameter and ¾″ deep, sealed at the top with a thin rubber membrane, is inserted between the leaf cup and the fixed resistance to respond to changes in air pressure in the system. A constant reduced pressure equivalent to about one cm. of water is applied to the open end of the fixed resistance. The magnitude of this pressure is controlled by

the depth to which the spill-over tube is inserted into the liquid in the pressure regulator.

If more air enters through the leaf into the leaf cup than is removed through the capillary resistance, the pressure within the system increases somewhat, causing the tambour membrane to rise. If less air enters through the leaf than is removed through the capillary resistance, the pressure decreases and the tambour membrane falls. Since the stomates control the passage of air through the leaf, the pressure changes reflect the movements of the stomates. As the stomates close, entrance of air is restricted and finally stopped, the pressure decreases and the tambour falls. As the stomates open, air enters the system, the pressure increases, and the tambour rises. The movements of the tambour membrane are transmitted by a
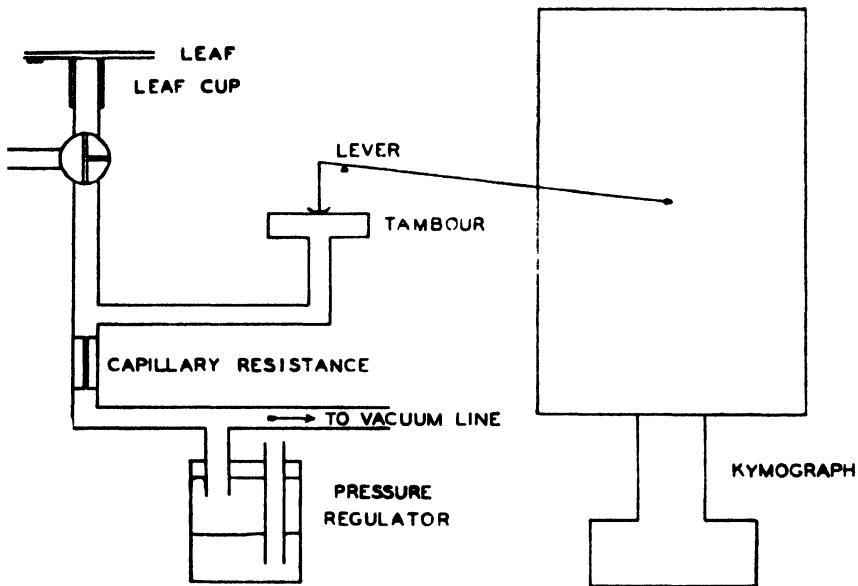
FIGURE 1

Fig. 1. Diagram of resistance porometer showing relation of various parts of system.

multiplying lever to smoked paper on a kymograph drum and a record is thus obtained.

The proper size of capillary resistance can only be ascertained by experimenting. If, as suggested by Gregory and Pearse (4), the thermometer tubing used for resistance is cut into segments with lengths in the ratio of 1:2:2:5:10, then a range of resistances from 1 to 20 can be obtained. Student thermometers, cut to a basic unit of 1 cm., were used. A glass T-tube was attached to both ends of each capillary segment. The tubes were then connected by rubber tubing to form a chain consisting of a piece of capillary alternating with a T-tube. Such an arrangement leaves an open end of a T-tube on either side of each piece of thermometer tubing. If these open ends are then joined by a piece of rubber tubing, they form a series of by-passes around each piece of thermometer tubing. However, if one of the

by-passes is closed with a metal clip, the air must pass through the capillary tubing. Thus by regulating the air through either the by-pass or the capillary tube, the total resistance in the circuit may be changed from 1 to 20 without allowing any outside air into the system. If too large a resistance is used, the tambour will register normal pressure while the stomates are still partly closed because air is not being removed as rapidly as it enters. If too small a resistance is used, the tambour will register reduced pressure even when the stomates are wide open because air is being removed more rapidly than it enters. The system is most sensitive when the fixed capillary resistance is equal to the resistance offered by the leaf, but since the leaf resistance varies greatly in the course of a day such a relation cannot be maintained. The proper resistance is one with which the change in pressures between fully open and completely closed stomates is within the total pressure range of the system.

In practice this is most easily attained by adjusting the resistances while the plant is under conditions which generally promote maximum stomatal opening; i.e., a high light intensity, fairly high humidity, and a temperature of between 25° and 30° C. Starting with maximum resistance in the line, the resistance is reduced, until the writing arm moves and then holds steady indicating slightly less than maximum pressure.

The leaf cups which are made of thin wall glass tubing, can vary somewhat in size, cups ranging from 0.9 cm. to 2.5 cm. in diameter having been used successfully. As many as six of the smaller cups, two on each of three plants, have been successfully connected on one recorder, thus giving the average behavior of the three plants. If the edges of the cups are ground, the cement will adhere better than to a smooth surface. The lower ends of the small cups are drawn out enough to permit directly connecting them to small bore rubber tubing. A one-hole stopper containing a piece of glass tubing is inserted in the bottom of the large cups to enable them to be connected to tubing. The volume of the cups and connecting tubing should be kept as small as possible to limit the volume of air in the system, thereby decreasing thermometer effects and increasing the speed of reaction to changes in stomatal aperture.

It is frequently difficult to seal leaves to the leaf cups. Numerous adhesives have been tried with varying success. Anderson found a mixture of approximately 1 part beeswax, 2 parts rosin, 2 parts lanolin and 1 part grafting wax fairly satisfactory for attaching leaf cups to cotton leaves. The writer found that if latex is first exposed to the air until it thickens to the consistency of thick cream it can be used to form a tough, air-tight and non-toxic seal. The edge of the leaf cup is covered with a layer of latex and held in contact with the leaf by means of a light weight until the latex is dry. Leaves of plants growing out-of-doors have been sealed to leaf cups with latex for more than a month without injury. Of course, the leaves must be supported in such a way that movements caused by wind will not

LEAF CLIP

TO
POROMETER
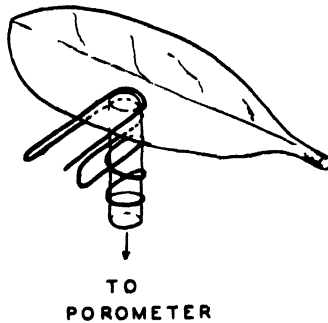
FIG. 2. Leaf clip for fastening leaves to porometer cup.

tear them loose from the rigidly fastened leaf cups. In addition to fastening the branches to supports, it is often helpful to hold the leaf in contact with the cup by means of a wire support as shown in figure 2.

The fulcrum of the magnifying lever was constructed from parts obtained from a cheap alarm clock. The clock was dismantled and all the gears except the balance wheel removed. Wooden dowels whose diameter was equal to the inside diameter of ordinary drinking straws were notched and cemented on opposite sides of the balance wheel similar to two spokes projecting from the same diameter. A straw with a gravity writing point was attached to one of the dowels. A weighted straw was attached to the other dowel to act as a counter balance. A hole was drilled in one of the spokes of the wheel into which could be inserted a free swinging arm which
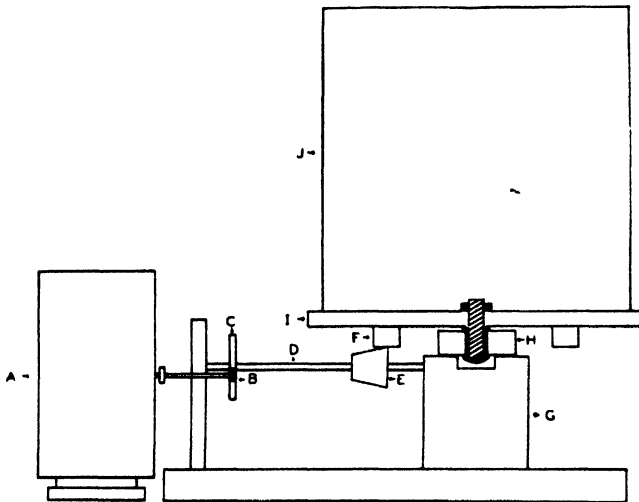
FIG. 3. Diagram showing construction details of kymograph. A, Big Ben clock; B, eight-tooth gear soldered to hour arm; C, forty-eight-tooth gear; D, connecting rod of capillary glass tubing; E, Rubber stopper; F, sponge rubber flange with circumference of four times E; G, kymograph support; H, roller skate bearing; I, kymograph drum platform; J, kymograph drum.

rested on the tambour. Two bolts were soldered to the top of the frame of the clock. The frame was then suspended by means of two screws from a metal L. By adjusting the screws, the writing point of the lever may be set to rest along any portion of the kymograph drum.

The construction of the kymograph is explained in figure 3. Although the construction was simple and very inexpensive, requiring only one ball-bearing roller skate wheel in addition to some gears obtained from the alarm clock, the kymograph was quite accurate and reliable. Eight kymographs were constructed, no one of which varied more than five minutes in twenty-four hours. Regular glazed kymograph paper is most suitable although
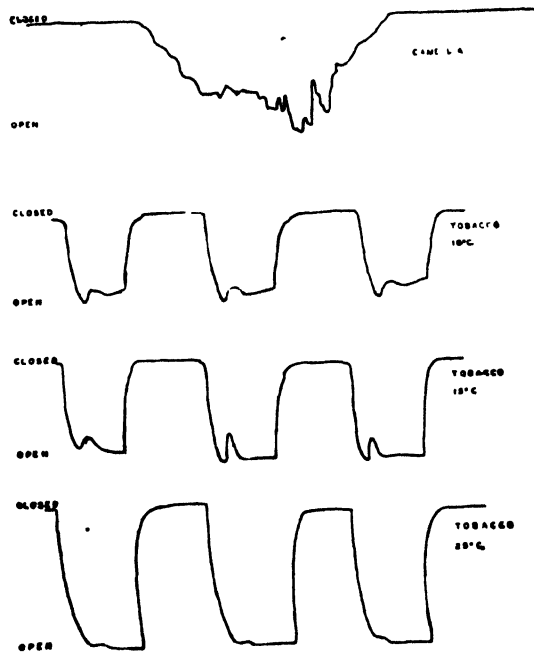
FIG. 4. Tracings of actual records; (a) twenty-four-hour record of Camellia under natural conditions; (b) records of tobacco under successive four hour light and dark periods at three different temperatures.

legible records were obtained using paper taken from better grade magazines. The drum, on which has been fastened a sheet of kymograph paper, is smoked over an ordinary wing tip burner whose gas supply has been bubbled through a wash bottle containing benzene. This procedure gives a very smoky flame and is best performed under a hood. After a record has been obtained, the paper is removed from the drum and fixed in a dilute solution of shellac.

Figure 4 shows tracings of actual records obtained from plants growing under both controlled and natural conditions. The results of scores of such records will be published in a later paper.

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CULTURE CONDITIONS AND THE DEVELOPMENT OF THE PHOTOSYNTHETIC MECHANISM. V. INFLUENCE OF THE COMPOSITION OF THE NURIENT MEDIUM

JACK MYERS

(WITH TWO FIGURES)

Significant effects of light intensity on the cellular and photosynthetic characteristics of *Chlorella pyrenoidosa* have been described in preceding papers (**6, 7**). These studies required a more or less arbitrary choice of other culture conditions, *e.g.*, light quality, temperature, carbon dioxide concentration, composition of the nutrient medium, and the population density at which cultures are maintained in the continuous-culture apparatus. Considered herein are the effects of various changes in composition of the nutrient medium and in the carbon dioxide concentration. The problems are: (1) whether the concentrations of various components of the medium are optimal and (2) whether there may be significant effects of small changes that might be induced as a reaction in response to other variables.

This investigation depends upon the use of a continuous-culture apparatus, previously described (**5**). The data to be presented demonstrate the usefulness of the apparatus in studies on nutrient deficiencies in micro-organisms.

Procedure

Chlorella pyrenoidosa was grown in a modified Knop's solution in two units of the continuous-culture apparatus. The Knop's solution contained 0.010 M $MgSO_4$, 0.012 M KNO_3, 0.009 M KH_2PO_4, 13.3×10^{-5} M $Fe_2(SO_4)_3$, 56.0×10^{-5} M sodium citrate, and 1.0 ml./l. each of the A_5 and B_6 solutions of ARNON (**1**). The A_5 solution contained 0.5 parts per million B, 0.05 p.p.m. Mn and Zn, 0.02 p.p.m. Cu, and 0.01 p.p.m. Mo; the B_6 contained 0.01 p.p.m. each of V, Cr, Ni, Co, W, Ti. The medium was prepared from stock solutions of the major salts purified by the adsorption procedure of STOUT and ARNON (**8**) and adjusted to pH 5.0.

The culture conditions adopted for reference were the same as those used previously, *i.e.*, a population density of 0.6–0.7 cmm. cells/cc., 4.4% carbon dioxide, and a temperature of 25° C. A light intensity of 25 fc as provided by tungsten lumiline bulbs was chosen because, as shown by the previous work, this gives cells with a maximum capacity for photosynthesis.

In the continuous-culture apparatus a photometric device controls the addition of fresh medium to the culture suspension at just such a rate that the density of population is maintained approximately constant. Samples of the culture suspension are harvested at 24-hour intervals, leaving a known amount of inoculum for the next interval. Because of the characteristics

of the apparatus, logarithmic growth is continuously maintained. Rate of growth is then expressible in terms of the constant k in the equation $\log \frac{N}{N_0} = kt$. In applying the equation N_0 is expressed as the milliliters of inoculum at the beginning of each interval and N as the total milliliters (ml. inoculum + ml. sample) at the end of each interval, t, of one day.

A new technique for changing the composition of the culture medium was introduced. Growth in the complete nutrient medium was maintained until growth rate, population density, and photosynthetic characteristics of the harvested cells were constant over a period of several days. Thereupon a new bottle of culture medium with a changed (or zero) concentration of some one component (e.g., iron) was inserted. As growth continued the culture medium in the chamber changed (was diluted) in respect to the varied component. Population density was held constant, and growth expressed itself only in volume of fresh medium added each day. The actual concentration of the varied component at any time could therefore be calculated. An assumption required is that the distribution of the component between cells and fluid was constant and independent of the concentration in the fluid. This is probably not entirely valid and in this respect the quantitative estimation of the actual concentrations is rendered uncertain for large changes. However, the interpretation of the results of a change in concentration or deficiency of any component is in no way affected.

Photosynthetic characteristics were studied manometrically by the Warburg technique at a temperature of 25° C. as previously described (7). Cells were immersed in Knop's solution (pH 4.5) saturated with 4% carbon dioxide and the rate of oxygen evolution calculated on the basis of an assimilatory quotient of – 0.90. Cell volumes were determined on duplicate aliquots by centrifuging in Van Allen thrombocytocrit tubes as previously described (6). Rates of photosynthesis could then be expressed in cmm. O_2/hr./cmm. cells. The rate of photosynthesis measured under conditions of light and carbon dioxide saturation is herein described as the *capacity for photosynthesis*.

Results

Comparison of population densities of 0.5 cmm. to 1.5 cmm. cells/cc. has revealed no significant differences in the subsequent photosynthetic behavior of the cells. At the higher population densities the growth rate is somewhat lower, however, a result of increased mutual shading and slightly lower effective light intensity. For subsequent work, therefore, a density of 0.5 cmm. to 0.7 cmm. cells/cc. has been adopted as a convenient working range.

EFFECTS OF MAJOR SALT CONCENTRATION

Data of an experiment designed to find the effects of changing the major salt concentration by the dilution technique described above are presented (table I). A culture was grown for a period of six days on Knop's solution

TABLE I

EFFECTS OF THE MAJOR SALT CONCENTRATION

DAY	RELATIVE CON-CENTRATION AT END OF DAY	GROWTH* RATE	CAPACITY FOR[†] PHOTO-SYNTHESIS	pH OF SUSPENSION
2–6	1.0	0.47	47	5.9
Insert 2X concentration of major salts				
7	1.65	0.45		
8	1.88	0.46		
9	1.95	0.43	46	
10	1.98	0.44	47	5.7
Insert zero concentration of major salts				
11	0.68	0.46		5.9
12	0.23	0.47	46	
13	0.089	0.43	47	7.0
14	0.046	0.28	19	7.0

$*\left(\log \dfrac{N}{N_0}\right)$ 24 hrs.

† cmm. O_2/hr./cmm. cells.

at its usual concentration. At the end of the sixth day a new bottle of medium was inserted containing twice the usual concentration of the major salts. From the amount of daily inoculum and daily harvest the relative concentration calculated for the end of each day is expressed in the second column. By the tenth day the major salt concentration had nearly doubled without appreciable effect on growth rate or the capacity of the cells for

TABLE II

EFFECTS OF DEPLETION OF IRON

DAY	FE CONCENTRA-TION AT END OF DAY	GROWTH RATE	CAPACITY FOR PHOTOSYNTHESIS	CHLOROPHYLL CONCENTRATION*
1	Insert Knop's containing zero Iron; original concentration had been 13.3×10^{-5} M			
7	0.049×10^{-5}	0.41	42	9.6
8	0.020	0.39	43	
9	0.0097	0.32	37	9.7
10	0.0044			
11	0.0021	0.34	38	8.2
12	0.0010	0.31	33	
13	0.00049	0.31	23	6.3
14	0.00025	0.30	18	
Insert Knop's containing 0.133×10^{-5} M Iron				
15	0.048			
16	0.096	0.36	43	
17	0.128	0.41	42	9.1

* In arbitrary units/cmm. cells (5).

photosynthesis. A bottle of medium was then inserted containing zero concentration of the major salts. Continued dilution of the major salt concentration finally gave rise to a reduced rate of growth and capacity for photosynthesis. In respect to these characteristics, however, it is apparent that the cells were insensitive to the major salt concentration over approximately a twenty-fold range. In the light of earlier work by MYERS (4), the development of the relatively high pH of 7.0 probably indicates a complete removal of nitrate from the medium on days 13 and 14 of the experiment.
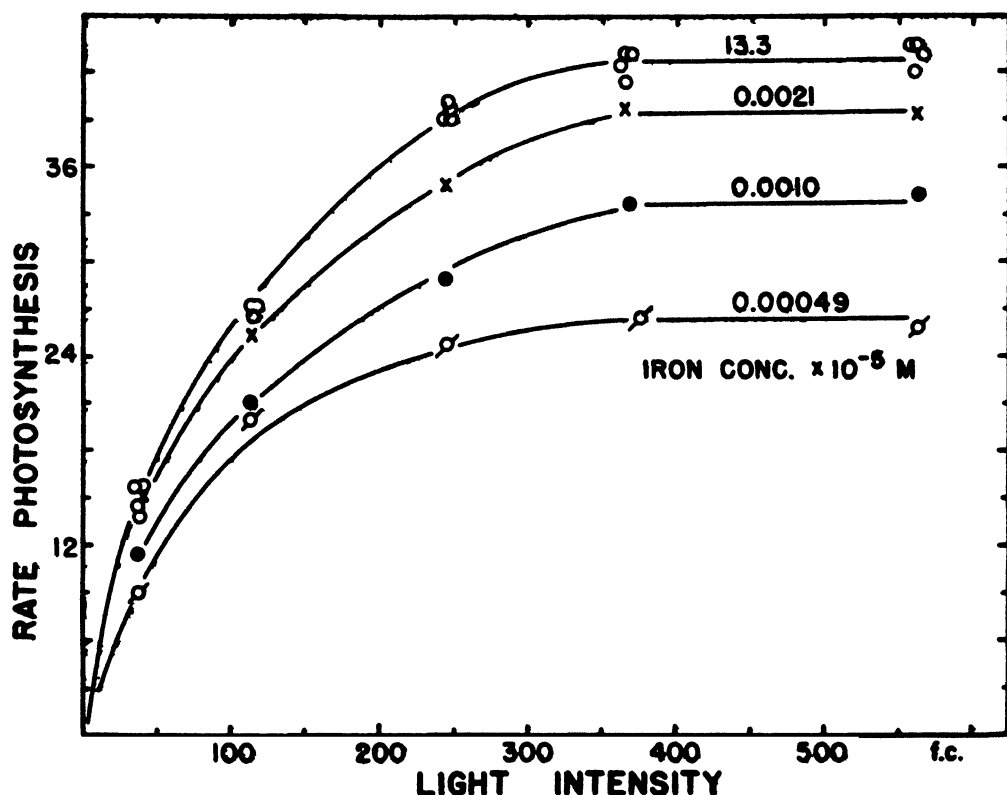
FIG. 1. Light intensity curves of photosynthesis for cells harvested on days 1, 11, 12, and 13 of the experiment described (table II). Values on the curves represent calculated iron concentrations at the time of harvesting. Rate = cmm. O_2/hr./cmm. cells measured in Knop's + 4.4% CO_2.

EFFECTS OF IRON CONCENTRATION

Results of the dilution technique applied to the study of iron concentration are given (table II). Growth rate, capacity for photosynthesis, and chlorophyll concentration were but slightly affected during the 270-fold drop in iron concentration over a six-day period. Further dilution of iron was accompanied by a decrease in all three of the cellular characteristics examined. Effects of iron depletion are shown in terms of the light intensity curves of photosynthesis for cells harvested at days 1, 11, 12, and 13 and labelled with the calculated iron concentrations prevailing at the time of harvesting (fig. 1). Any one of the lower curves can be made

to fit approximately the upper curve by multiplying all points by a single factor. No apparent differential effects of iron concentration on the "photochemical." and "dark" processes are detectable in this experiment.

EFFECTS OF MICROELEMENT CONCENTRATIONS

With the usual iron concentration of 13.3×10^{-5} M it was impossible to demonstrate any effects of depletion of the A_5 or B_6 microelement solutions by the dilution technique. The iron concentration was thereupon reduced to 0.133×10^{-5} M. Effects of depletion of the A_5 solution could then be noted (table III). It is apparent (as previously noted by others) that iron

TABLE III

EFFECTS OF DEPLETION OF THE A_5 SOLUTION
(Original concentration = 1.0 ml./liter; contains B, Cu, Mn, Mo, Zn;
Iron concentration 0.133×10^{-5} M)

DAY	FRACTION OF ORIG. CONCENTRATION AT END OF DAY	GROWTH RATE	CAPACITY FOR PHOTOSYNTHESIS	CHLOROPHYLL CONCENTRATION
2–5	1.0	0.45	47	9.3
Insert Medium Containing Zero A_5				
6	0.35	0.45	47	..
7	0.13	0.43	44	
8	0.078	0.22	11	5.3

salts without special purification contain appreciable amounts of the requisite microelements. No further attempts have been made to determine critical levels or effects of deficiency of any of the individual microelements of the A_5 solution since this is beyond the scope of the present work. Even with the lowered iron concentration, no effects of depletion of the B_6 solution could be observed.

EFFECTS OF HYDROGEN ION CONCENTRATION

The Knop's solution used for growth throughout these experiments was initially adjusted to pH 5.0. This gave a pH of about 5.8 in the culture suspension. Changes in initial pH such that the culture suspension was maintained at pH 5.2 or pH 6.7 revealed no significant effects on the photosynthetic behavior of the harvested cells. However, at higher pH iron slowly precipitated despite the added citrate, and at lower pH the suspensions foamed badly and the algae tended to deposit on the upper walls of the chamber. A pH of culture of about 5.8 seemed to be an optimum for practical operation.

EFFECTS OF CONCENTRATION OF CARBON DIOXIDE

Comparative data on the effects of provision of air and 4.4% carbon dioxide in air on the subsequent photosynthetic behavior of the cells are

TABLE IV

EFFECTS OF CARBON DIOXIDE CONCENTRATION

CARBON DIOXIDE PROVISION	GROWTH RATE	CAPACITY FOR PHOTOSYNTHESIS
4.4%	0.45	43
Air	0.26	34

shown (table IV and fig. 2). The actual concentrations of carbon dioxide available to cells in the growth chambers is not known. Changes of from 2% to 5% carbon dioxide in the influent gas mixture have been found to be without effect on the growth rate of subsequent photosynthetic activity of the cells. It is therefore assumed that aeration with 4.4% carbon dioxide provides a saturating concentration for photosynthesis. In a culture aerated with air, however, the calculated rate of carbon dioxide utilization approaches the rate of provision in the influent air. The effective concentration in the culture must be considerably less than the initial 0.03% in the influent air. This is borne out by the observation that in air growth rate is dependent on rate of aeration (carefully controlled in the above experiment) while in 4.4% carbon dioxide growth rate is independent of aeration rate.

It has already been shown (7) that at 25 fc growth rate is proportional to light intensity and therefore probably determined by rate of photosyn-
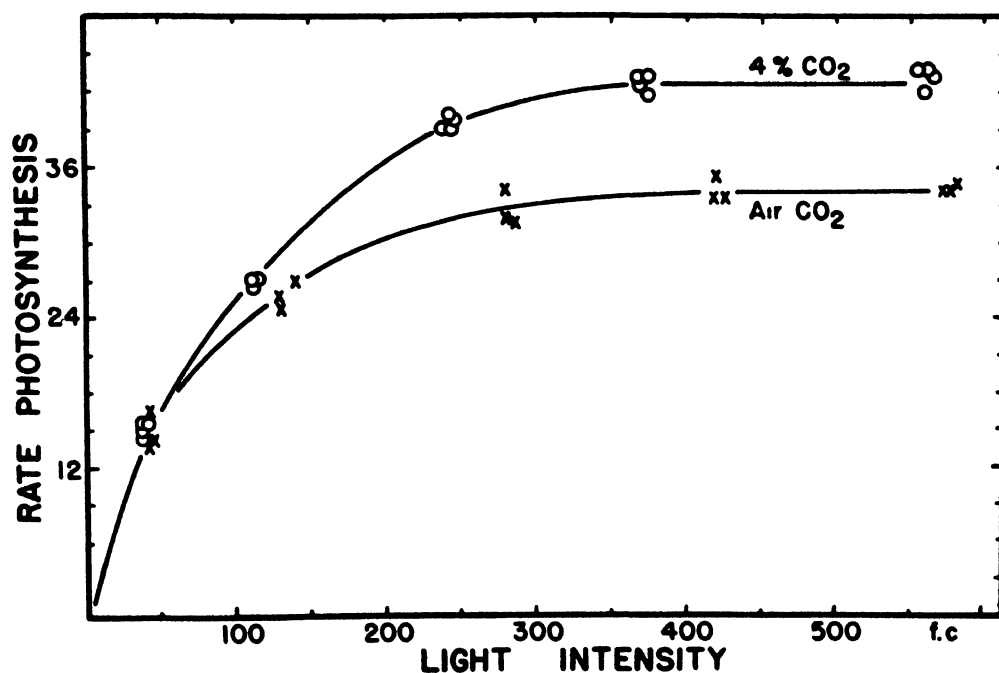
FIG. 2. Light intensity curves of photosynthesis for cells grown at different concentrations of carbon dioxide. Values on the curves indicate carbon dioxide composition of the influent air of the growth chambers. Rate = cmm. O_2/hr./cmm. cells measured in Knop's + 4.4% CO_2.

thesis. EMERSON and GREEN (2) have shown that in *C. pyrenoidosa* rate of photosynthesis is independent of carbon dioxide concentration down to less than 0.05%. On these grounds also one may predict a limiting rate of provision of carbon dioxide on aeration with air. The problem of rate of provision of carbon dioxide has been discussed previously (4).

Limited provision of carbon dioxide during growth gives rise to cells with a lowered capacity for photosynthesis at high light intensities (Blackman rate) as indicated (fig. 2). On the other hand, limiting carbon dioxide has no effect on the subsequent rates of photosynthesis at low light intensity (photochemical rate).

Further work on effects of limited carbon dioxide provision had been planned. Unfortunately, cells grown under these conditions tend to cling to the sides of the chamber so that further work along this line has been abandoned due to the technical difficulties involved.

Discussion

The techniques described above offer a new approach to the general problem of evaluating the critical concentration of any substance required in the nutrition of a microorganism. Comparison with the conventional method is illustrated by consideration of the work of HOPKINS and WANN (3) on the iron requirement for Chlorella. By addition of graded amounts of iron they were able to determine: (1) the minimum concentration at which growth would take place and (2) the amount of iron per culture required to allow development of a maximum crop of cells. The second type of determination is a rather general one although it merely indicates how great the initial concentration must be to prevent its becoming a limiting factor in the growth of a culture. In contrast to either type of information afforded by this method, the technique introduced above allows an estimation of the minimum concentration needed to support a maximum rate of growth. Quantitative comparison of these data with those of Hopkins and Wann is impossible since they used a different strain of Chlorella, a different concentration of citrate, and were then unaware of microelement requirements which must have been provided in their iron. It is clear, however, that quite different physiological characteristics are studied by the two methods.

It is also apparent that the continuous-culture technique is neatly adapted to studies of the specific effects of nutrient deficiencies or of inhibitors on microorganisms. Any given nutrient or inhibitor can be maintained continuously in a culture at any desired level and cells withdrawn periodically for physiological study.

The work herein reported was undertaken primarily to continue a survey of the effects of various culture conditions on photosynthetic characteristics of Chlorella. It has been found, in regard to the composition of the nutrient medium, that the concentrations originally chosen more or less arbitrarily give cells with optimum growth rate and capacity for photosynthesis. Small

changes in these concentrations, which might be induced by changes in other conditions, are without appreciable effects.

Summary

1. *Chlorella pyrenoidosa* was grown in a continuous-culture apparatus. Composition of the nutrient medium, carbon dioxide concentration, and population density were studied for their effects on growth and subsequent phytosynthetic behavior.

2. Growth and photosynthetic behavior are relatively insensitive to large changes in the composition of the medium. The major salt concentration may be varied about 20-fold, iron 270-fold, A_5 microelement concentration 3-fold, hydrogen ion 30-fold, all without effect.

3. Limited provision of carbon dioxide during growth yields cells with a lowered capacity for photosynthesis.

4. The data presented demonstrate the usefulness of the continuous-culture technique in studies on nutrient deficiencies in microorganisms.

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A FURTHER INVESTIGATION OF THE REPLACEMENT OF BORON BY INDOLEACETIC ACID[1]

Robert MacVicar and W. E. Tottingham

It has been reported (2) that low concentrations of indoleacetic acid in the nutrient solutions supplied to cotton seedlings will partially replace boron for growth in cultures deficient in this element. This finding constitutes a fundamental advance in the elucidation of the role of boron in the metabolism of the plant. The further implication of auxins in respiration (1) and growth indicates that boron might function through some relationship with these enzyme systems. It was believed desirable, therefore, to attempt to confirm this work and extend it to other and more suitable experimental plants.

Experimental data

Plants were grown in washed silica sand in varnished clay pots. Seedlings of suitable size, germinated in either sand or soil, were transplanted into sand which had been thoroughly leached with distilled water. The following experimental plants were used: cotton, unknown variety; sunflower, var. Russian Mammoth; tomato, var. Early Baltimore; tobacco, var. Maryland Mammoth; and soybean, var. Manchu no. 3. Four replications of each treatment were made with the exception of the series harvested on June 21, 1941. In this case only three replications were employed.

The composition of the nutrient solutions was as follows:

KH_2PO_4	0.0018 M
K_2HPO_4	0.0012 M
$Ca(NO_3)_2$	0.0042 M
$CaCl_2$	0.0011 M
$Mg(NO_3)_2$	0.0021 M
$MgSO_4$	0.0011 M
NH_4NO_3	0.0042 M

During periods of low light intensity ammonia nitrate was omitted from the solution. Minor elements were included in the concentrations recommended by Hoagland (3). In series 1 the solution was supplied twice daily by a manually operated air pressure type subirrigation system. In series 2, approximately 500 ml. of nutrient solution was supplied manually each day. Unbalanced solution was removed by weekly leaching with distilled water. The indoleacetic acid was supplied in the nutrient solution at a level of 1 p.p.m. when the subirrigation culture was employed. In the latter series it was added separately and cumulatively each day in such amounts as to pro-

duce a concentration of 0.2 p.p.m. in the solution, assuming saturation of the sand.

Normal greenhouse conditions prevailed. During periods of high light intensity, plants were kept in whitewashed houses having intensities of approximately 1000 foot candles. This was similar to the intensity employed by EATON (2). Summer temperatures varied between 65° to 90° F. during the day and 60° to 80° F. during the night. Winter temperatures were 65° to 70° F. and 60° to 65° F., respectively. Photoperiods were normal during the summer months and were lengthened to 16 hours by means of incandescent lamps during the winter.

TABLE I

SERIES 1.   EFFECT OF INDOLEACETIC ACID ON GROWTH OF PLANTS IN THE PRESENCE AND ABSENCE OF BORON IN THE NUTRIENT MEDIUM

PLANT, TREATMENT, AND DATE OF SAMPLING	DAYS OF IAA TREAT-MENT[2]	WET WEIGHT OF TISSUE PER PLANT		DRY WEIGHT OF TISSUE PER PLANT		DRY MATTER		TOP / ROOT
		TOP	ROOT	TOP	ROOT	TOP	ROOT	
		gm.	*gm.*	*gm.*	*gm.*	%	%	
June 26, 1941								
Sunflower, CN[1]		41.7	15.9	3.73	0.80	8.9	5.0	4.6
" CN–B		15.3	6.2	1.61	0.40	9.5	6.5	4.0
" CN + IAA[1]	15	40.0	16.9	3.17	0.95	7.9	5.6	3.5
" CN + IAA–B	15	18.2	6.7	1.65	0.44	9.1	6.6	3.8
Cotton, CN		21.8	8.9	2.27	0.52	9.6	5.9	4.4
" CN–B		8.1	3.8	1.08	0.32	10.3	8.4	3.4
" CN + IAA	15	19.4	6.95	2.13	0.36	11.0	5.2	5.9
" CN + IAA–B	15	12.3	8.3	1.14	0.47	9.3	5.6	2.4
Soybean, CN		28.6	18.9	4.26	1.27	14.4	6.7	3 0
" CN–B		18.9	15.8	2.83	1.05	15.0	6.0	3.8
" CN + IAA	15	32.6	20.9	4.71	1.35	14.4	6.5	2.9
" CN + IAA–B	15	15.8	13.2	2.72	0.95	15.5	7.2	2.9
Dec. 13, 1941								
Tobacco, CN		253.6	32.8	20.80	2.68	9.2	8.2	7.8
" CN–B		101.0	15.4	9.99	1.52	10.9	9.9	6.6
" CN + IAA	65	252.7	41.7	21.98	3.61	10.4	8.7	8.3
" CN + IAA–B	65	167.7	21.6	14.42	1.86	7.5	8.6	7.8

[1] CN, complete nutrient; IAA, indoleacetic acid.
[2] Concentration 1.0 p.p.m. in nutrient solution.

Under the conditions used, boron deficiency symptoms appeared in those plants not receiving this element without regard to the presence or absence of indoleacetic acid. No compensatory action was noted either in the time of onset or degree of intensity of deficiency symptoms. This observation was substantiated by anatomical observations of the stems.[2] Examination of sections of stem taken at the fourth internode from the stem tip showed the necrotic areas typical of boron deficiency in the region of the first formed xylem and the surrounding xylem parenchyma cells regardless of the presence of indoleacetic acid in the culture.

Examination of the data showed that in only one instance was there any

[2] Appreciation is expressed to B. ESTHER STRUCKMEYER, Department of Horticulture, University of Wisconsin, who prepared and examined these sections.

TABLE II

SERIES 2. EFFECT OF INDOLEACETIC ACID ON GROWTH OF PLANTS IN THE PRESENCE AND ABSENCE OF BORON IN THE NUTRIENT MEDIUM

PLANT, TREATMENT AND DATE OF SAMPLING	DAYS OF IAA TREATMENT[2]	WET WEIGHT OF TISSUE PER PLANT			DRY WEIGHT OF TISSUE PER PLANT			DRY MATTER			TOP/ROOT RATIO
		LEAF	STEM	ROOT	LEAF	STEM	ROOT	LEAF	STEM	ROOT	
		gm.	gm.	gm.	gm.	gm.	gm.	%	%	%	
October 17, 1941											
Sunflower, CN		15.9	30.9	17.3	2.26	2.62	1.63	14.2	8.5	9.4	3.3
" CN–B		7.5	13.6	6.2	0.81	0.91	0.49	10.8	6.7	7.9	3.5
" CN+IAA[1]	40	14.5	29.9	14.0	2.04	2.82	1.32	14.1	9.5	9.4	2.8
" CN+IAA–B	40	7.5	18.6	9.7	1.26	1.22	0.75	16.8	6.6	7.7	3.2
January 16, 1942											
Tomato, CN		40.7	31.7	20.9	4.59	3.79	1.72	11.2	8.6	8.2	4.9
" CN–B		30.1	21.8	13.1	2.87	1.99	1.25	9.6	9.1	9.5	4.9
" CN+IAA	30	37.9	31.9	22.1	4.00	3.23	1.67	10.5	10.1	7.6	4.2
" CN+IAA–B	30	31.9	21.2	11.2	2.90	1.79	0.96	11.0	11.8	8.6	4.8
Sunflower, CN		14.2	38.9	9.6	1.63	2.69	0.68	11.5	6.9	7.1	6.4
" CN–B		8.9	12.3	5.4	0.85	0.73	0.24	10.5	5.9	4.5	6.6
" CN+IAA	30	16.0	37.1	13.2	1.88	3.94	0.95	8.5	9.1	7.2	6.1
" CN+IAA–B	30	7.5	13.1	5.3	0.79	0.81	0.23	10.5	5.8	4.4	6.5

[1] CN, complete nutrient; IAA, Indoleacetic acid.
[2] Concentration 1.0 p.p.m. in solution in October 17, 1941 sample; added daily and cumulatively at 0.2 p.p.m. in January 16, 1942 sample.

significant increase in the dry weight of boron deficient plants receiving indoleacetic acid (tables I and II). In this instance, the tobacco harvested on December 19, 1941, was significantly higher when indoleacetic acid was supplied. It had been noted, however, that the symptoms of boron deficiency appeared somewhat earlier in those plants receiving indoleacetic acid and more secondary tissue was produced from developing axillary buds. Renewed growth in such circumstances is frequently observed in tobacco that has become severely deficient (5). It is believed that the somewhat greater mass of tissue produced by plants receiving indoleacetic acid may have been principally due to the earlier appearance of initial symptoms. No significant correlations of moisture content or root-top ratios to the presence or absence of either boron or indoleactic acid were found.

Discussion

It is recognized that the technique employed in this study differed in several major respects from that of EATON (2). He used unaerated solution culture and produced symptoms of boron deficiency at a much earlier stage of growth than is possible under normal conditions in sand cultures. Whether any protective action of indoleacetic acid in conditions producing boron deficiency is more efficient in the seedling than in the more mature plant is, as yet, undetermined. The concentration of indoleacetic acid, moreover, was higher in the sand culture experiments.

It may be that other environmental factors are involved in the differences of results obtained. Eaton noted that the protective action of indoleacetic acid was largely nullified by high light intensities (2), and it may be that this effect is more pronounced in the older plants. It has also been observed that the severity of boron deficiency may be accentuated by high levels of calcium (6). The solution employed in this study contained higher levels of calcium ion than did that used by Eaton in his study. HOAGLAND and SNYDER (4) have suggested that the degree of aeration of nutrient solutions may be related to the severity of boron deficiency. Suitable conditions for repeating these experiments under more rigidly controlled conditions of light intensity and other environmental factors are not available to us. Elucidation of a possible relation between boron and the auxins, however, might well provide valuable insight into the metabolic function of both.

Examination of the data indicates, however, that under the conditions employed any appreciable replacement of boron by indoleacetic acid in growing plants was absent. Whether or not it occurs under more suitable and limited environmental conditions is still unknown.

Summary

No significant alleviation of boron deficiency by indoleacetic acid was noted in sand culture under normal greenhouse conditions with tomato, cotton, sunflower, soybean, and tobacco plants.

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GERMINATION OF *ASYSTASIA GANGETICA* L. SEED WITH SPECIAL REFERENCE TO THE EFFECT OF AGE ON THE TEMPERATURE REQUIREMENT FOR GERMINATION[1]

ERNEST K. AKAMINE

Introduction

Freshly harvested seed of *Asystasia gangetica* L., an ornamental shrub, does not germinate well under ordinary conditions. This seed normally requires a storage period of several months at room temperature before it germinates. The purpose of this investigation is to determine the requirements for germination of the seed at different ages.

The literature on the use of alternating temperatures to break the dormancy of seeds is voluminous, and no attempt will be made here to refer to all of the reported cases. HARRINGTON (2) extensively studied the use of alternating temperatures to increase the germination of seeds of grasses and garden crops. MORINAGA (3) increased the germination of Bermuda grass seed with alternating temperatures. TOOLE (4) also used alternating temperatures to improve the germination of other grass seeds. It has been the experience of the author that the germination of seeds of some of the grass species grown on Hawaiian ranges is materially improved by the use of alternating temperatures (1).

Methods

Seeds used in the investigation reported herein were harvested at maturity from Asystasia plants grown on the Hawaii Agricultural Experiment Station grounds. Curing was effected by storing the seeds at room temperature conditions until they were used for germination tests.

Germination tests were conducted in Petri dishes using filter paper moistened with distilled water. Ovens and cold storage chambers were used for temperature variations. Temperatures employed consisted of: 6.6°–10.2°, 15.0°, 20.8°–28.0° (room temperature), 29.9°–33.2°, and 36.0° C. and combinations of these for the alternating temperatures. For temperature alternations, the Petri dishes with the seeds therein were transferred from one temperature to another at regular intervals. The schedule of daily alternating temperatures listed in table I was followed, and except in some unavoidable cases, the daily 8-hour period extended from 8 A.M. to 4 P.M. and the 16-hour period from 4 P.M. to 8 A.M. the following day. The number of seeds used for the germination tests varied from 25 to 100 seeds per treatment. In a few cases where the seed supply was sufficient, replicated tests were conducted.

[1] Published with the approval of the Director as Technical Paper no. 155 of the Hawaii Agricultural Experiment Station.

Results

Results of many tests to increase the germination of freshly harvested seed of Asystasia indicated that temperature has a marked influence on germination and that the alternating temperature treatment is the most effective. At continuous temperatures the dormant seed does not germinate as well as at alternating temperatures. Whatever temperature treatment is necessary to break the dormancy, a minimum temperature of approximately 20° C. must be provided for actual germination.

Removal of the seedcoat of normally dormant seed produces a germination of approximately 75% at room temperature, whereas the intact seed produces no germination or at best a very low germination. The maximum germination produced by this treatment, however, is not so high as the

TABLE I

SCHEDULE OF DAILY ALTERNATING TEMPERATURE TREATMENTS

TEMPERATURE ALTERNATION	PERIOD AT LOWER TEMPERATURE	PERIOD AT HIGHER TEMPERATURE
°C.	hours	hours
6.6–10.2 to 15.0	8	16
6.6–10.2 to 20.8–28.0 (room temperature)	8	16
6.6–10.2 to 29.9–33.2	8	16
6.6–10.2 to 36.0	8	16
15.0 to 20.8–28.0	8	16
15.0 to 29.9–33.2	8	16
15.0 to 36.0	8	16
20.8–28.0 to 29.9–33.2	16	8
20.8–28.0 to 36.0	16	8
29.9–33.2 to 36.0	16	8

germination (about 90% and over) obtained by the use of optimum alternating temperatures.

After performing a number of germination tests with seeds harvested at different times, it became apparent that seeds of different ages respond differently to the various temperature treatments. Attempts were then made to determine the temperature requirement for maximum germination at different ages (since harvest). A complete tabulation of all of the data is not included in this report, but a compilation of some of the results obtained in these trials is recorded in table II and a résumé of all tests conducted on different lots of seed harvested at various times is presented in table III which shows the temperature requirement for germination of seed of different age groups.

From tables II and III it is seen that for germination after one week of curing, the fresh seed requires a severe temperature treatment, and as the seed ages, this treatment becomes less and less severe until finally the seed requires no special treatment and germinates at room temperature (20.8°–28.0° C.). When the seed is only two to seven days old, it requires an

TABLE II

EFFECT OF TEMPERATURE VARIABLES ON THE GERMINATION OF *Asystasia gangetica* L.
SEED OF DIFFERENT AGES

AGE OF SEED SINCE HARVEST*	TEMPERATURE TREATMENT		No. OF SEEDS USED	GERMINATION IN TWO WEEKS
days		°*C.*		%
	Alternating	6.6–10.2 to 20.8–28.0 (room temperature)	100	51.7
	"	6.6–10.2 to 29.9–33.2	100	67.2
	"	6.6–10.2 to 36.0	100	51.7
	"	15.0 to 20.8–28.0	100	86.0
7	"	15.0 to 29.9–33.2	100	88.0
	"	15.0 to 36.0	100	63.0
	Continuous	20.8–28.0	100	1.7
	Alternating	20.8–28.0 to 29.9–33.2	100	84.7
	"	20.8–28.0 to 36.0	100	93.2
	Alternating	6.6–10.2 to 29.9–33.2	75	91.9
	"	6.6–10.2 to 36.0	75	100.0
	Continuous	20.8–28.0	75	0.0
14	Alternating	20.8–28.0 to 29.9–33.2	75	30.0
	"	20.8–28.0 to 36.0	75	60 0
	Continuous	29.9–33.2	75	0.0
	"	36.0	75	26.1†
	Alternating	6.6–10.2 to 29.9–33.2	100	97.4
	"	6.6–10.2 to 36.0	100	68.4
	"	15.0 to 20.8–28.0	100	93.0
20	"	15.0 to 29.9–33.2	100	94.0
	"	15.0 to 36.0	100	76.0
	"	20.8–28.0 to 29.9–33.2	100	44.7
	"	20.8–28.0 to 36.0	100	50.0
	Alternating	6.6–10.2 to 20.8–28.0	100	94.7
	"	6.6–10.2 to 29.9–33.2	100	90.0
	"	6.6–10.2 to 36.0	100	84.2
28	Continuous	20.8–28.0	100	5.2
	Alternating	20.8–28.0 to 29.9–33.2	100	79.7
	"	20.8–28.0 to 36.0	100	87.5
	Continuous	29.9–33.2	100	12.1
	"	36.0	100	29.3†
	Alternating	15.0 to 20.8–28.0	100	99.0
58	"	20.8–28.0 to 29.9–33.2	100	56.0
	"	20.8–28.0 to 36.0	100	35.0
	Alternating	15.0 to 20.8–28.0	50	87.0
84	Continuous	20.8–28.0	50	8 0
	Alternating	20.8–28.0 to 29.9–33.2	50	84.0
	"	20.8–28.0 to 36.0	50	92.0
	Continuous	20.8–28.0	25	16.7
	Alternating	20 8–28.0 to 29.9–33.2	25	91.7
112	"	20.8–28.0 to 36.0	25	89.0
	Continuous	29.9–33.2	25	0.0
	"	36.0	25	41.7†
	Alternating	15.0 to 29.9–33.2	25	91.7
	Continuous	20.8–28.0	25	96.8
140	Alternating	20.8–28.0 to 29.9–33.2	25	100.0
	Continuous	29.9–33.2	25	41.7
	"	36.0	25	50.0†

* Seed cured and stored at room temperature conditions.
† Even though germination at 36.0° C. was superior to that at 29.9°–33.2° C., it is probable that the higher temperature is excessive for germination of the seeds continuously exposed to it because injury to the seeds was apparent at this temperature.

alternation between room temperature and 36.0° C. for germination. When the seed is 8 to 14 days old, it requires a more drastic treatment—an alternation between the lowest and the highest temperatures used. This difference in the germination requirement in the two age groups is probably due to the fact that in one case the seed—especially the seedcoat—is not fully cured, thus requiring a less severe treatment, whereas in the other, the seed is fully cured, thus requiring more extreme temperatures in the alternation. When the seed is older than 14 days, it requires less and less drastic treatment for germination as the seed ages. Finally when the seed is 135 days old and older it germinates normally at room temperature.

TABLE III

THE OPTIMUM TEMPERATURE CONDITIONS FOR MAXIMUM GERMINATION AT THE VARIOUS AGES OF THE SEED OF *Asystasia gangetica* L.

AGE OF SEED SINCE HARVEST*	TEMPERATURE REQUIRED FOR MAXIMUM GERMINATION	
days		*°C.*
2 to 7	Alternating	20.8–28.0 (room temperature) to 36.0
8 to 14	"	6.6–10.2 to 36.0
15 to 26	"	6.6–10.2 to 29.9–33.2
27 to 36	"	6.6–10.2 to 20.8–28.0
37 to 82	"	15.0 to 20.8–28.0
83 to 88	"	20.8–28.0 to 36.0
89 to 134	"	20.8–28.0 to 29.9–33.2
135 to 168	Continuous	20.8–28.0 or alternating 20.8–28.0 to 29.9–33.2

* Seed cured and stored at room temperature conditions.

Discussion

Since removal of the seedcoat promotes germination at room temperature, though not to a degree of alternating temperatures, it seems that the cause of dormancy in the freshly harvested seed of Asystasia mainly lies in the nature of the seedcoat. The difference in germination percentage resulting from the removal of seedcoat and the use of alternating temperatures is probably due to the more favorable influence of the alternating temperatures on the respiration process of the seed. The seedcoat readily allows passage of water, but it probably restricts the passage of gases involved in respiration. Removal of the seedcoat might eliminate this obstruction to gas movement.

A higher germination with alternating temperatures than with continuous temperatures was obtained in dormant Asystasia seed (table II). HARRINGTON (2) states that the beneficial effect of alternating temperatures on germination is due not to the specific effect of the extreme temperatures of the alternation or to the mean temperature of the alternation but to the changes in the temperatures. Since removing the seedcoat allows for germination, it seems probable that the alternating temperatures render the intact seedcoat more permeable to essential materials required for germination.

Since the severity in the temperature alternation necessary for germination decreases with the increase in age of the seed (tables II and III), it seems that normally the change in permeability comes about through the aging (after 7 days) of the seedcoat. Perhaps simultaneously the after-ripening process proceeds as the seed ages. Thus with the aging of the seed, the severity of the temperature treatment required for germination decreases until finally when the seedcoat is no longer an obstruction to essential materials the seed germinates under ordinary conditions.

Summary

1. Freshly harvested seed of *Asystasia gangetica* L. does not germinate well at room temperature.

2. After a period of approximately 135 days in ordinary storage, it germinates readily at room temperature.

3. Seeds between the ages of 8 and 134 days require an alternation of temperatures for germination, the severity of which is related to the age of the seed—the younger the seed the more severe the required treatment and *vice versa*.

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THE EFFECT OF ULTRAVIOLET LIGHT ON SUBSEQUENT RIPENING OF THE FRUIT OF THE TOMATO (*LYCOPERSICON ESCULENTUM*)

ERSTON V. MILLER[1] AND HAROLD A. SCHOMER

Introduction

While conducting physiological studies on the development of color in Florida oranges, it was observed that frequently midseason fruits developed a rich orange color when they were growing on the northwest side of the tree and a pale yellow color when growing on the southeast side. It appeared that this difference in color of the fruits was in some way related to the unequal exposure to sunlight and quite possibly the result of different amounts of ultraviolet light received. A few tests were therefore made to determine the effects of ultraviolet irradiation on pigment development in fruits in the hope that the information obtained might contribute to a better understanding of color development in citrus fruits. Preliminary studies indicated that ultraviolet irradiation of harvested, mature-green oranges retarded the rate of degreening of these fruits but did not in any way effect the yellow pigments. The conditions in the laboratory in these experiments were not quite comparable to those existing in the grove because oranges do not show an increase in carotenoid pigments after removal from the tree. However, it was not practicable to subject the oranges to ultraviolet irradiation while they were still attached to the tree. The only alternative was to select a fruit which had been shown to increase in carotenoid pigments after removal from the parent plant. It was for this reason that subsequent experiments were conducted with fruits of the tomato (*Lycopersicon esculentum*).

Materials and methods

Mature green tomatoes were sorted into lots of 10 to 20 each, the fruits in each lot being comparable in regard to size and degree of maturity.[2] One lot (original sample) was analyzed immediately for chlorophyll and carotenoid pigments. Other lots were given ultraviolet irradiation by means of a "Sterilamp"[3] placed 18 inches above the fruits. The position of the

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[2] The term "mature green" is applied to green tomatoes that will ripen after removal from the vine. They consist of fruits that have advanced from the dark green to the light green stage. However, since these changes are progressive and since the fruits are selected entirely by appearance, the term actually represents a range rather than a specific stage of maturity. In making up the experimental lots an attempt was made to select fruits of a uniform color.

[3] According to Westinghouse Company, manufacturers of the "Sterilamp," energy is emitted by this lamp in wave lengths ranging from 2537 to 5780 Angstrom units, with the lower wave lengths representing 83.03% of the total. The intensity at a distance of

tomatoes was reversed during the period of exposure in order to permit both stem-end and blossom-end to receive an equal amount of irradiation. A stream of air directed over the irradiated fruits prevented the temperature from rising above that of the control lot. Following the irradiation the tomatoes were held in the 70° F. room until the majority of the control fruits were fairly well colored. In most cases the fruits were given a preliminary treatment with ethylene gas, in order to shorten the time required for ripening.

Total chlorophyll and total carotenoid pigments were determined by a method reported previously (2, 3). Essentially, the method consisted of extraction of the fresh pulp with acetone, transferring the extract to petroleum ether, and purifying. The chlorophyll was separated by saponification and determined colorimetrically by means of a Clifford photometer. The total carotenoids (remaining in the petroleum ether) were likewise determined colorimetrically. Total acidity was determined by extracting 100 grams of the pulp with boiled distilled gater, filtering, and titrating the filtrate with standard NaOH. Details of this method have been published elsewhere (1).

Results

The results obtained in the first experiment are presented in table I. It will be noted that at the end of the ripening period all of the fruits in lot 2 (no irradiation) were either pink or red. Lot 3 (3 hours' irradiation) contained seven pink and no red fruits, while lot 4 (5 hours' irradiation) contained six pink fruits and one red fruit. The others in these two lots were either green or turning. That the exposure to ultraviolet light retarded ripening was evidenced by both the failure to lose the green color and the failure to produce the orange red pigments.

In order to express the data quantitatively the fruits were analyzed for chlorophyll and carotenoid pigments. These data are also presented in table I. The sample taken at the beginning of the experiment (lot 1) contained 2.85 milligrams of chlorophyll and 0.417 mg. of carotenoid pigments per 100 grams of flesh. After the two days' ripening period the control lot contained 0.55 mg. of chlorophyll and 1.662 mg. of total carotenoids per 100 grams. The two irradiated lots had not lost as much chlorophyll nor had they produced as great a quantity of carotenoid pigments as the control lots during the same time. Lot 3 (irradiated 3 hours) contained 1.12 mg. of chlorophyll and 1.125 mg. of total carotenoids. Lot 4 (five hours' irradiation) contained 1.00 mg. of chlorophyll and 1.140 mg. of total carotenoid pigments per 100 grams of flesh. In other words, the control lot had produced at least 45% more carotenoid pigments than had the irradiated lots.

Table II shows the effect of ultraviolet irradiation on the subsequent

18 inches would be equal to 150.19 microwatts per square centimeter. The shortest measurable rays in solar radiation are approximately 2900 Angstrom units. It is thus apparent that the radiations from the Sterilamp were not equivalent to the ultraviolet radiation in sunlight.

TABLE I

EFFECT OF ULTRAVIOLET IRRADIATION ON SUBSEQUENT RIPENING OF TOMATO FRUITS

| LOT | PERIOD OF IRRADIATION | NUMBER OF FRUITS IN EACH COLOR GROUP | | | | TOTAL CHLORO-PHYLL | TOTAL CAROTENOIDS |
		GREEN	TURN-ING	PINK	RED		
						mg./100 gm.	mg./100 gm.
1	0 (orig. sample)	10	0	0	0	2.85	0.417
2*	0 (control)	0	0	5	5	0.55	1.662
3	3 hours	2	1	7	0	1.12	1.125
4	5 hours	3	0	6	1	1.00	1.140

* Record made of lots 2, 3, and 4 after two days in ethylene.

ripening of Rutgers tomatoes. It is significant that there were no fully ripe tomatoes in any lot except the one receiving no irradiation. In fact, there were no pink fruits in any irradiated lot except number 4 (two hours' irradiation), which contained eleven. Some of the fruits in this lot may have been more mature than others when sorted. Except for those in lot 4 none of the irradiated fruits had progressed beyond the "green" or "turning" stage at the end of the ripening period.

Table III shows the results of a second experiment conducted with Rutgers tomatoes in Florida. The original sample contained 2.13 mg. of chlorophyll and 0.225 mg. of total carotenoids per 100 grams of flesh. After two days in ethylene and five days in air at 70° F. the control lot had lost all of its chlorophyll and had produced 5.24 mg. of total carotenoids per 100 grams of flesh. As in previous experiments the fruits in the irradiated lots lost less chlorophyll and produced less carotenoid pigments than did those in the control lots. The values for these two lots expressed as mg. of pigment per 100 grams of flesh are as follows: Lot 3 (two hours' irradiation), 1.19 mg. of chlorophyll and 2.120 mg. of carotenoids; lot 4 (four hours' irradiation), 1.00 mg. of chlorophyll and 0.657 mg. of carotenoids.

TABLE II

EFFECT OF ULTRAVIOLET IRRADIATION ON SUBSEQUENT DEVELOPMENT OF COLOR IN GREEN TOMATOES*

| LOT | PERIOD OF IRRADIATION | NUMBER OF FRUITS IN EACH COLOR GROUP | | | |
		GREEN	TURNING	PINK	RED
1	0 (orig. sample)	18	0	0	0
2	0 (control)	7	5	2	4
3	1 hour	8	10	0	0
4	2 hours	2	5	11	0
5	3 hours	9	9	0	0
6	4 hours	14	4	0	0

* Record on all but original sample made after two days in ethylene and five days in air at 70° F.

It seemed desirable at this time to determine whether ripening processes, other than development of color, were affected by the ultraviolet light. Accordingly the pulp of the fruit in the last experiment was analyzed for total acids. The control lot contained 0.540% acid; the lot receiving two hours' irradiation, 0.630%; and the lot which was irradiated four hours contained 0.755%. These data indicate that exposure to ultraviolet light retards the loss of acidity in green tomatoes.

TABLE III

EFFECT OF ULTRAVIOLET IRRADIATION ON TOTAL CHLOROPHYLL, CAROTENOIDS, AND
ACIDS IN GREEN TOMATO FRUITS*

LOT	PERIOD OF IRRADIATION	CHLOROPHYLL	CAROTENOIDS	ACID
		mg./100 gm.	*mg./100 gm.*	*%*
1	0 (orig. sample)	2.13	0.225	....
2	0 (control)	......	5.240	0.540
3	2 hours	1.19	2.120	0.630
4	4 hours	1.00	0.657	0.755

* Record on all but original sample made after two days in ethylene and five days in air at 70° F.

Discussion

When a mature green tomato is removed from the vine it will, under satisfactory conditions, lose its green pigment and develop a good red color. The green pigment disintegrates and a considerable quantity of the orange-red pigments is manufactured. Ultraviolet irradiation has been shown to arrest both the destruction of the green and the production of the orange-red pigments. Smith (4) has reported that lycopene formation in tomatoes is favored by protection from the light whereas exposure to light is conducive to the development of carotene. Since lycopene constitutes the bulk of the carotenoid pigments in ripe tomatoes, it is apparent in these experiments that ultraviolet irradiation inhibits formation of lycopene.

A maturing orange, while still on the tree, is much like a tomato in that the chlorophyll is decreasing while the carotenoid pigments are increasing. Once an orange is removed from the tree, however, the green pigments disappear, revealing the yellow pigments that have been manufactured up to this point, but there is no increase in carotenoid pigments after the orange has been harvested. This is the reason that the oranges that have been degreened with ethylene usually have a pale yellow and not a deep orange color.

Chromatographic analyses in this laboratory have indicated that while there is a certain amount of carotene in orange rinds, the deep orange color is due to lycopene. This applies to the highly colored oranges of the Mandarin type, to the sweet oranges grown in the northern part of Florida, and even to midseason oranges produced on the northwest side of the trees.

Evidence that ultraviolet light in the sun prevents the formation of pig-

ments in the exposed fruits on the southeast side of the tree is therefore only circumstantial and should not be taken too seriously, but it suggests that this is a field which should be explored for both practical and scientific reasons.

Summary

Exposing mature green fruits of the tomato (*Lycopersicon esculentum*) to the rays of an ultraviolet lamp tended to retard subsequent ripening. The irradiation retarded the loss of chlorophyll, the development of carotenoid pigments, and the reduction of acidity.

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INFLUENCE OF HIGH, MEDIUM, AND LOW SOIL MOISTURE ON GROWTH AND ALKALOID CONTENT OF *CINCHONA LEDGERIANA*

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(WITH THREE FIGURES)

Introduction

Soil moisture in Cinchona growing regions may fluctuate greatly with seasonal changes in rainfall; prolonged droughts are experienced almost annually. On the other hand, during the peak of the rainy season the same soils may remain at or near saturation for extended periods. For example, at the Toro Negro National Forest in Puerto Rico, where Cinchona is being grown experimentally, the 23-year-average rainfall for two-week periods from January 1 to March 25 was 2.04 inches with frequent periods of drought. In the current year only 0.21 inch fell during January. From August 13 to November 18 rainfall averaged 5.69 inches for two-week periods. The average total annual rainfall for 23 years was 96.37 inches (4).

The above extremes in soil moisture may be expected to exert marked influence on the growth and composition of Cinchona. HEINICKE, BOYNTON and REUTHER (3) demonstrated that a flooded (poorly aerated) soil produced symptoms in the apple typical of boron deficiency when the soil was not deficient in that element. It was also indicated that lack of oxygen in the soil interfered with the absorption of nitrogen and potassium. WAGER (6) showed that avocado plants were more susceptible to disease if grown in a flooded soil than if watered normally.

During the first year from seed, Cinchona plants are primarily herbaceous. It is during this tender stage that soil moisture is most likely to be a critical factor affecting growth. Since the plants are grown in palm-leaf-covered nurseries during this stage it is fairly easy to correct a deficiency of soil water by irrigation, but more difficult to prevent an excess during the rainy season. Cinchona nursery plantings have been observed which were apparently healthy during three-fourths of the year, but with the advent of heavy daily rains during the peak of the rainy season the plants developed symptoms typical of nutrient deficiencies and many succumbed to fungus attack.

The experiment reported here was designed to study the effect of low, medium, and high levels of soil moisture on growth and alkaloid content of young *Cinchona ledgeriana* seedlings under controlled conditions.

Materials and Methods

In March 1946 seedlings of *Cinchona ledgeriana* were transplanted to soil in concrete benches located in three air-conditioned greenhouse chambers, described elsewhere (7). The bench in each chamber was divided into three randomized plots each about 25 inches square. The plots were separated by a double partition of boards spaced 4 inches apart. One hundred and forty-five pounds of a uniform mixture of soil were placed in each plot. The soil consisted of equal parts of clay loam, silica sand, peat moss, and leafmold. The wilting point was 9.5% moisture and the field capacity approximately 36%. The wilting coefficients were determined as the percentage of moisture remaining in the soil at the time of permanent wilting of soybean plants growing in the soil. With this information on soil moisture characteristics it was possible to establish treatments as follows: (1) Low soil moisture ranging between 9 and 13%, (2) medium soil moisture ranging between 18 and 24%, and (3) high soil moisture ranging between 30% and field capacity.

The Cinchona seedlings were selected for uniformity and were about 4 inches high when transplanted. Sixteen seedlings, five inches apart, were planted to each plot.

The temperature in all chambers was maintained at 75° F. during the day and 65° F. at night. In the previous experiment (7) this temperature was found to be the most favorable for growth of *C. ledgeriana*. Relative humidity was maintained as high as possible by continually spraying the floors with water. During the middle of the day relative humidity usually dropped to 60 or 70% but most of the time it ranged between 80 and 95%.

All plots were watered uniformly at regular intervals through March and most of April. On April 25 all plots were watered to field capacity and then allowed to dry gradually until the wilting percentage was approached on June 14. Samples for soil moisture determinations were taken at weekly intervals during the drying period and from these data the approximate rate of water loss in each plot was calculated. Since the weight, volume, wilting point, and field capacity of the soil were the same in each plot it was possible to determine fairly closely the amount of water needed to bring the soil moisture content to the desired levels.

At the time the differential soil moisture treatments were begun on June 21 the soil moisture in all plots was near the wilting point. Different volumes of water were added to the various plots in order to raise the level of soil moisture to the maximum percentage for each treatment range. For example, sufficient water was added to the low-moisture plots to bring the level to 13%, to the medium-moisture plots to bring the level to 24%, and to the high-moisture plots to bring the level to field capacity. The approximate volume of water necessary thereafter for each plot was determined from tables prepared from data obtained as described above. The required volume of water for each plot was applied uniformly over the entire area

with a sprinkling can. At intervals of 7 to 10 days soil plugs $\frac{1}{2}$ inch in diameter were taken from four locations in each plot. Each plug extended to the entire depth of the plot so that the moisture percentage obtained represented a fair average of the volume of soil in which the roots were distributed. After the sample was taken the holes were filled and a new location sampled at the next date. The percentages of soil moisture were determined from the loss in weight of soil dried for 24 hours at 105° C. From the soil moisture data thus obtained it was possible to estimate the approximate time required for a plot to reach the point when water should be needed. Also, maintenance of the desired soil moisture percentages was facilitated by the fact that the rate of water loss by transpiration and evaporation was fairly uniform due to controlled temperature and humidity in the chambers.

The experiment was terminated on January 6, 1947, at which time the soil was washed from the roots. Data on height and fresh and dry weights were obtained and the top-root ratio was calculated. The roots and stems were analyzed for quinine and total alkaloids by the method described by LOUSTALOT and PAGÁN (5). The leaves were analyzed for ash, nitrogen, phosphorus, potassium, calcium, and magnesium by the methods outlined by DROSDOFF and PAINTER (1).

Results

EFFECT OF SOIL MOISTURE ON GROWTH AND ALKALOID CONTENT

Figure 1 shows a weekly record of the soil moisture percentage in the three plots of replication Number 1 at the various sampling dates from June 18, 1946, to January 4, 1947. These data are typical of those from
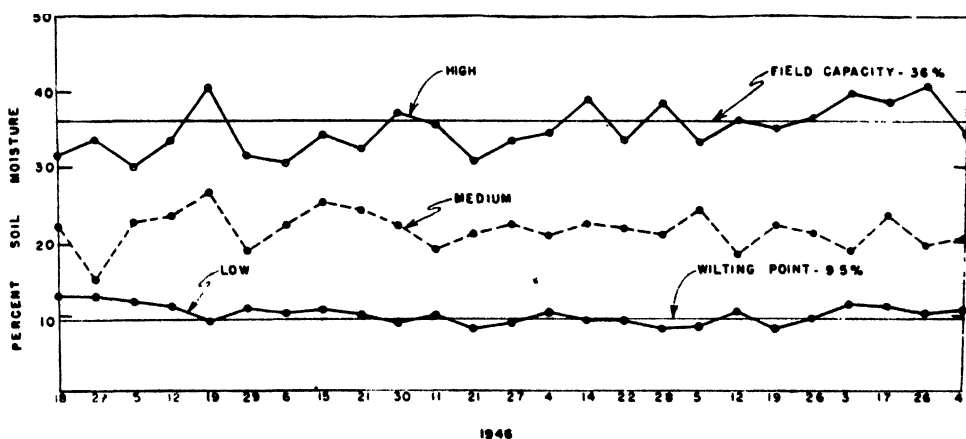
FIG. 1. A typical soil moisture record for plots maintained at high-, medium-, and low-moisture levels. Growth of Cinchona seedlings in these plots is shown in figure 2.

the other two replications. There were some rather wide fluctuations in soil moisture at first, particularly in the medium-soil-moisture plots, but with additional experience it was possible later to control the soil moisture within the designated range. The moisture level that fluctuated the least and consequently was the easiest to control was the low-soil-moisture treat-

FIG. 2. Left to right: typical Cinchona plants grown in plots maintained at high, medium, and low levels of soil moisture. Note crinkling and spotting of tip leaves of plant grown in high soil moisture on the left (see also figure 3).

ment. Only sufficient water was supplied in this treatment to keep the soil moisture about 1 to 3% above the wilting percentage of 9.5 Although on several occasions the soil moisture dropped below the wilting percentage, there was no pronounced evidence of wilting. This was probably due to the fact that the temperature in the chambers was cool and the humidity
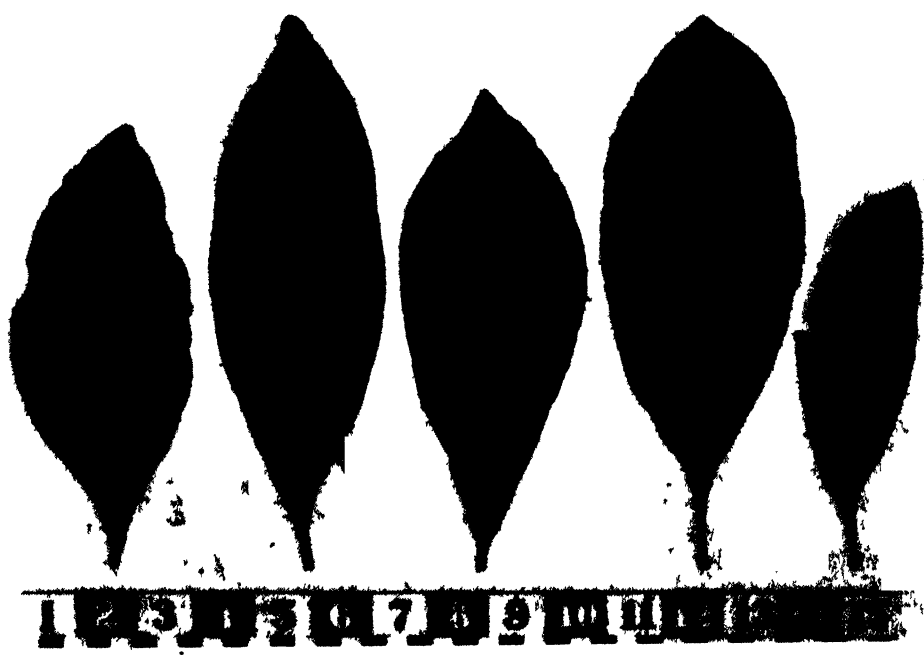
FIG. 3. Leaves of Cinchona showing from left to right progressive stages of disease symptoms on plants subjected to continuous high soil-moisture conditions.

relatively high, a situation similar to that occurring in most commercial cinchona plantations.

About three months after the treatments were started, small yellow spots which later became necrotic appeared systematically between the veins of leaves of plants in the high-soil-moisture plots (figs. 2 and 3). This disorder which somewhat resembles magnesium deficiency (2) or manganese toxicity in other plants later became more pronounced and occurred on nearly all the plants in the high-soil-moisture treatment. Some of the plants lost as much as three-fourths of their foliage. A few of the plants in the medium- and low-soil-moisture plots showed these symptoms but the incidence was less than one-tenth that in the high-soil-moisture plots. This disorder often has been observed on young cinchona trees in the station planting at Toro Negro at 3,200 feet elevation. It is not certain if the disease is pathological or physiological or a combination of both, but it is clear that a high-soil-moisture condition is associated with it.

It is evident from data in table I that seedlings grown at the low-soil-

TABLE I

Survival and Growth of *Cinchona ledgeriana* under low, medium, and high soil moisture conditions

SOIL MOISTURE TREATMENT	AV. HEIGHT	AV. FRESH WEIGHT	AV. DRY WEIGHT	DRY MATTER	SUR-VIVAL	TOP-ROOT RATIO
	cm.	*gm.*	*gm.*	%	%	
Low (9 to 13%)	38.9	23.9	6.3	26.4	95.8	6.3
Medium (18 to 24%)	56.4	45.8	10.2	22.2	97.9	8.4
High (30% to field capacity)	64.3	45.7	9.9	21.7	95.8	7.0

moisture level made less growth as measured by height and fresh and dry weights than seedlings grown at medium- and high-soil-moisture conditions. These differences were significant at the 1% level. As might be expected, the percentage of dry matter in the plants grown in low soil moisture was significantly higher than that in plants from the two other treatments. The differences in height and fresh and dry weights between plants grown in medium and high moisture were not statistically significant.

Survival was good and fairly uniform in all treatments; it was slightly but not significantly higher in the medium-soil-moisture plots. The top-root ratios of the plants grown in medium and high soil moisture were not significantly different from each other, though both were significantly greater than the top-root ratio of plants grown under low-soil-moisture conditions.

The total alkaloid and quinine-sulfate content of the roots and stems are given in table II. The roots of plants grown under medium- and high-soil-moisture conditions contained significantly higher total alkaloid and quinine sulfate than the roots of plants grown under low-soil-moisture con-

TABLE II

TOTAL ALKALOID AND QUININE SULFATE IN ROOTS AND STEMS OF *Cinchona ledgeriana* GROWN UNDER LOW-, MEDIUM-, AND HIGH-SOIL-MOISTURE CONDITIONS

SOIL MOISTURE TREATMENT	TOTAL ALKALOIDS		QUININE SULFATE	
	STEMS	ROOTS	STEMS	ROOTS
	%	%	%	%
Low (9 to 13%)	1.90	3.49	0.99	1.77
Medium (18 to 24%)	1.87	4.00	0.83	1.84
High (30% to field capacity)	1.78	3.87	0.96	1.90

ditions. There was no statistically significant differences in the total alkaloid content of the stem nor of the quinine sulfate content of the stems and roots of the plants in the three treatments.

EFFECT OF SOIL MOISTURE ON MINERAL COMPOSITION

From the data presented in table III it is apparent that the soil moisture treatments had a moderate effect on the mineral composition of the leaves. The percentages of ash, calcium, and magnesium were somewhat higher in the leaves from plants grown under low-soil-moisture conditions than in those from plants grown under medium- and high-soil-moisture conditions. Leaves from plants grown under high-soil-moisture conditions contained the lowest percentage of ash, calcium, and magnesium, while leaves of plants from the medium-soil-moisture plots were intermediate with respect to these constituents. The reverse situation was true, however, with respect to the nitrogen, phosphorus, and potassium contents of the leaves. Leaves from plants grown in low-soil-moisture plots contained significantly the least of these elements. There was no significant difference between the contents of these elements in leaves from plants grown in high- and medium-soil moisture.

TABLE III

MINERAL COMPOSITION OF *Cinchona ledgeriana* LEAVES FROM PLANTS GROWN UNDER HIGH-, MEDIUM-, AND LOW-SOIL-MOISTURE CONDITIONS*

SOIL MOISTURE TREATMENT	ASH	TOTAL NITROGEN	PHOS-PHORUS	POTAS-SIUM	CALCIUM	MAG-NESIUM
	%	%	%	%	%	%
Low (9 to 13%)	4.99	1.86	0.19	0.71	0.77	0.55
Medium (18 to 24%)	4.65	2.21	0.23	1.02	0.69	0.43
High (30% to field capacity)	4.23	2.35	0.25	1.09	0.57	0.41

* Expressed as percentage of dry weight.

Summary

1. Seedlings of *Cinchona ledgeriana* were grown in greenhouse chambers under high-, medium-, and low-soil-moisture conditions at Mayaguez, Puerto Rico. The temperature was automatically controlled and the humidity was maintained relatively high.

2. Leaves of seedlings grown under high-soil-moisture conditions developed necrotic spots between the veins and many of them eventually yellowed and abscissed. This disorder, which resembles magnesium deficiency or manganese toxicity in other plants, has been commonly observed in the station field plantings at Toro Negro National Forest, Puerto Rico.

3. Seedlings grown under low-soil-moisture conditions were significantly shorter with a lower top-root ratio and less fresh and dry weight, but a higher percentage dry matter than seedlings grown under either medium- or high-soil-moisture conditions.

4. Total alkaloid and quinine sulfate in roots of seedlings grown in medium and high soil moisture were significantly higher than those in roots of seedlings grown in low soil moisture.

5. Percentages of ash, calcium, and magnesium were highest in plants grown in low soil moisture and lowest in plants grown in high soil moisture. The nitrogen, phosphorus, and potassium contents, however, were significantly the least in plants grown in low soil moisture.

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DISTRIBUTION OF THIAMIN AND RIBOFLAVIN IN THE TOMATO PLANT[1]

FELIX G. GUSTAFSON

The vitamin content of plant material has been extensively studied during the last few years but these studies have dealt almost exclusively with single organs of a plant, such as fruits or other edible part, and only a few have been concerned with the distribution of these vitamins within the plant. The investigations that have had as their objective the determination of the quantity of a particular vitamin in different parts of a plant have, in general, shown that the young parts contain more vitamin than the older parts. LAMPITT, BAKER, and PARKINSON (8) found that ascorbic acid was present in highest concentration in young leaves and young tubers of the potato plant. RYTZ (9) found that in the pea plant there was a decrease in thiamin as the plant aged, the leaves always containing more than the stem, which had a higher concentration than the roots. Flower buds were on par with young leaves. BURKHOLDER and McVEIGH (3) observed that in corn seedlings the embryo leaves contained most thiamin and, as the leaves became older, there was a decrease. The same authors (4) found that thiamin, riboflavin, niacin, and B$_c$ (the antianemic factor) increased in the buds of trees as they opened but again there was a decrease as the leaves matured. On the other hand, BURKHOLDER and SNOW (5) noted that in forest trees the buds contained more thiamin than the leaves. BONNER (1) and BONNER and DORLAND (2) found that the youngest tomato leaves contained the highest concentration of thiamin, riboflavin, and pantothenic acid. There was also a gradient in the stem with the younger part having more vitamin than the older part. The roots had a higher concentration of riboflavin and pantothenic acid than any part of the stem, but thiamin was present in a higher concentration in the young stem than in the root.

The present work has as its purpose to extend the work on distribution of vitamins in the tomato plant.

Methods

The method developed by CONNOR and STRAUB (6, 7) for combined riboflavin and thiamin determination has been used throughout the work, with only some slight modifications necessary in the use of plant material. In this method the plant material is first homogenized and then extracted with 0.05 N H$_2$SO$_4$ solution at a temperature of boiling water for a period of one hour with occasional stirring. The extract is digested with clarase at

a pH of 4.5 for two hours at a temperature of 45° C. to break down the cocarboxylase which is a pyrophosphate ester of thiamin. Next the digest is centrifuged for 45 minutes at 2,500 rpm. Twenty-five milliliters of the clear solution is slowly dropped, first onto activated Decalso then onto Florisil; the former adsorbs the thiamin and the latter the riboflavin. The adsorbents are held in place by a small amount of glass wool in 15-milliliter special tubes, and the tubes are so arranged that the solution runs from the one with Decalso into the one with Florisil. After the solution has run through the tubes the adsorbents are washed with 35 ml. of hot distilled water. The tubes are next separated and the thiamin is eluted with hot 25% KCl to a volume of 40 ml. Five-milliliter aliquots are oxidized with alkaline potassium ferricyanide to thiochrome, which is taken up in 15 ml. of isobutyl alcohol. The isobutyl alcohol is separated by centrifugation, pipetted off, and any water removed with anhydrous sodium sulfate. The fluorescence of the thiochrome contained in the isobutyl alcohol is determined by a lumetron fluorimeter. The isobutyl alcohol, clarase, and perhaps the plant material itself, contains some material fluorescing in ultraviolet light; thus an unoxidized, as well as an oxidized, aliquot is run from each sample. The fluorescence of the unoxidized aliquot is subtracted from that of the oxidized aliquot and the difference is the fluorescence due to the thiamin present in the plant material. Repeated tests of the clarase have shown it to be free from thiamin. For standardization of the light intensity in the lumetron a quinine sulfate solution having a concentration of 27 mcg./ml. is used.

The adsorbed riboflavin is eluted with 20% pyridine-2% acetic acid solution and made up to a volume of 50 ml. Aliquots of 15 ml. are pipetted into brown Erlenmeyer flasks and the foreign substances which may also fluoresce are oxidized by 1% potassium permanganate, the excess of which is destroyed by 3% hydrogen peroxide. The riboflavin is determined in the lumetron by its yellow-green fluorescence in blue light. It has been found that the clarase contains some riboflavin and therefore a control is run with clarase and its fluorescence in blue light is subtracted from the plant sample to get the fluorescence due to the riboflavin in it. For standardization of the light intensity a 0.1 mcg./ml. solution of sodium fluorescein is employed. All of these determinations must be done in subdued light because riboflavin is destroyed by light.

Calculations are made by the use of standard curves obtained by the use of known concentrations of thiamin and riboflavin.

The plant material has been the John Baer tomato grown in the greenhouse. As the experiments have extended over a considerable period of time the plants have been exposed to different light conditions, but as no comparisons are made between different experiments this does not matter.

Results

In this investigation attempts have been made to gain information on the general distribution of thiamin and riboflavin in the tomato plant. In the

first few experiments the apical part of the plant, including the immature stem and leaves, and mature leaves were used. Later a more extensive examination of the plant was made in which leaves of four different ages, obtained from different levels of a tall plant, were employed. Both of these sets of experiments are recorded in Section One of table I. It will be noted that the immature leaves and stem apex contain the most thiamin and riboflavin, and there is a gradual decrease in the concentration of these vitamins

TABLE I

THIS TABLE IS A SUMMARY OF A NUMBER OF EXPERIMENTS IN WHICH VARIOUS COMPARISONS AS TO CONCENTRATIONS OF THIAMIN AND RIBOFLAVIN HAVE BEEN MADE. THE FIGURES DENOTE MICROGRAMS PER GRAM OF FRESH MATERIAL. DIFFERENT GROUPS OF EXPERI MENTS ARE REFERRED TO AS SECTIONS FOR READY REFERENCE TO THE TEXT

SECTION	DATE	PART OF PLANT USED	THIAMIN	RIBOFLAVIN
I	12–13	Immature leaves and stem	1.5 1.9	2.67 3.22
	12–20	Mature leaf, (only blade) Whole mature leaf	1.2 0.46	3.50 2.17
		Whole leaves from different parts of stem		
	5–4	a. Immature leaves	3.69 2.26	1.57 2.83
	5–6	b. Mature leaves—18 in. below *a*	1.07 0.92	1.47 1.82
		c. Mature leaves—15 in. below *b*	0.23 0.67	1.34 1.20
		d. Pale green with yellow markings	0.77 0.08	1.12 1.01
II	1–15	Only leaf blade—mature leaf	0.29 1.61	2.05 1.20
	2–11	Rachis and petiole	0.00 0.04	0.50 0.32
		Stem near leaf insertion	0.06 0.31	0.50 0.45
III	2–5	Apical part of plant	1.22 1.23	1.60 2.05
	6–8	Middle part of plant	0.46 0.27	0.86 1.01
		Roots	0.15 0.23	1.02 0.99
IV	4–13	Leaves	0.77 0.92	1.41 0.77
	4–15	Ripe fruits	0.73 0.46	0.53 0.48

as the leaves age. An apparent exception to this is the result for the experiment of May 4, in which the concentration of the thiamin is higher in the oldest leaves than in the ones just preceding them. The reason for this is that the oldest leaves used in this experiment were very old and some were partly dead with dry margins. This would increase the dry weight. Even under these conditions the riboflavin showed a steady decrease with the age of the leaves. These experiments are confirmations of BONNER's findings (1) some years ago for the tomato plant, and agree with investigations of other plants where it has been shown that the young parts of a plant are richest in vitamins.

In the experiment of December 13, Section One of table I, only the leaf blade was used of the mature leaf and the thiamin content was essentially the same as that of the immature leaf and stem and the riboflavin concentration was even higher, whereas in another experiment run a few days later the whole mature leaf was quite low in these vitamins, thiamin especially. This led to an investigation of the distribution within the leaf and the stem to which the leaf was directly attached. Section Two of table I shows two of these experiments; the second one was run 13 months later than the first one. It is to be observed that the rachis and petiole contain no or very little thiamin and less riboflavin than the blade. The thiamin concentration was very low in the first experiment and that may account for the lack of the vitamin in the rachis and petiole in this experiment; in the later experiment, however, the thiamin was quite high in the blade and yet the rachis and petiole were practically devoid of thiamin. The difference in the riboflavin concentration was somewhat less in the different parts. These experiments demonstrate clearly that the blade is richer in thiamin and riboflavin than either the rachis and petiole of the leaf or the stem to which the leaf is directly attached.

It was also desirable to investigate the roots. For this purpose plants grown in nutrient solution were used. The two experiments cited under Section Three of table I were made on plants 13–16 inches tall in the first experiment and 24–26 inches tall in the second experiment. The second lot of plants were about three months old. Taking the two experiments together the roots contain a little less thiamin than the leaves and stems of the middle portion of the plant and much less than the apical part, but the riboflavin is not essentially different in the roots and the middle portion, though here also the apical part is much richer in vitamin. The two experiments cited in table I were made four months apart and the results are essentially the same, though the plants were grown under different light and temperature conditions and the plants in the later experiment were more vigorous than those in the earlier one.

One more group of experiments was set up to examine the relation between vitamins and tissue. In this group of experiments immature leaves and stems were obtained from the main apex of plants of different age-groups. The results are recorded in table II. The youngest plants always contained the lowest concentration of both thiamin and riboflavin, but the former showed a much greater difference. It is very surprising that the youngest plants, which have the highest physiological activity, had the lowest vitamin content. This can be explained in a number of ways. One might consider that a precursor for the vitamin is built up slowly and in some other part of the plant and as the plant becomes larger it produces more of this substance which is then activated in the young part of the plant. It could also be considered that a larger and older plant has accumulated more stored material which is used in the synthesis of the vitamins. As the days get longer and lighter in the spring it takes less time for this ma-

TABLE II

This table shows a comparison of the concentration of thiamin and riboflavin in the tips (immature stems and leaves) derived from plants of different ages. Age of the plants is given in months at top of columns, and the amount of vitamin in micrograms per gram fresh material. The figures within the brackets below vitamin values are the heights of the plants in inches

Date	Thiamin					Riboflavin				
	1	2	3	4	5	1	2	3	4	5
1-10		0.03 (5)	0.75 (26-30)	1.12 (60-64)			1.36	1.74	1.87	
2-20	0.54 (5-6)		1.33 (22-23)	2.20 (50-60)		1.33		1.87	1.89	
4-20		1.11 (15-19)	1.84 (36-42)		2.61 (72-80)		1.55	1.63		1.34
4-22	1.30 (4-5)		2.45 (36-42)	2.26 (52-64)	2.53 (72-80)	1.54		2.24	2.40	1.68

terial to accumulate and in April it has reached a sufficient level in three-months-old plants, whereas that was not true in January when the days were short and dark.

Most of the data available on the tomato in the literature is on the fruit and as these experiments are concerned with the distribution in the whole plant a comparison between fruits and leaves has also been made. Section Four in table I shows two such experiments made in April. In one experiment the difference is very large but in the other it is small as to the thiamin concentration, but the riboflavin is always much more abundant in the leaves than in the fruits. Experiments made in January give a greater difference in both vitamins between the leaves and fruit than the later experiments. These findings are a little different from those reported by WILSON and WITHNER (10) who found that the thiamin concentration was always greater in the fruits than in the leaves and roots, but conversely the leaves had more riboflavin than the fruits. They used five varieties of tomato but not John Baer.

Discussion

While no experiments have been made to determine the effect of light on the formation of the thiamin and riboflavin there seems to be considerable evidence that light influences the synthesis of thiamin. In nearly all instances where determinations were made several months apart those made later when the days were longer and brighter had the higher concentration. This is specially well brought out in table II. No light measurements were made so naturally one cannot speak too authoritatively in this connection, but the general trend makes one suspect that light furthers the synthesis of thiamin, either directly or indirectly. The light does not seem to influence riboflavin nearly so much.

The great difference in thiamin and riboflavin concentration in the apical region of plants of different ages is very puzzling but as explained earlier it must be due to a larger amount of precursor produced in the larger plants. If this precursor or perhaps merely labile food material is conducted to the growing regions where the thiamin is synthesized the larger the photosynthesizing area the more of this material will be available. Undoubtedly there would be reached an age or size after which there would be no further increase as the capacity of the synthesizing region has also been reached. It will be seen from table II that as far as the riboflavin is concerned this seems to have been reached in plants three months old in the April experiments, though perhaps not in the thiamin synthesis. This view is further strengthened by the fact that as more light becomes available for photosynthesis the difference between the young and old plants becomes less.

There seems to be very little doubt as to the tissue in which the synthesis takes place. The leaves are much richer in vitamins than any other part of the plant and when the leaf is divided into blade, rachis, and petiole, the blade has practically all of the vitamin of the whole leaf. One might then think that there is a close relationship between photosynthesis and

thiamin synthesis, but that need not be true. Leaves are active growth centers and there is no reason why vitamin synthesis should not also take place there, even though it is not in any way connected with photosynthesis.

Summary

The distribution of thiamin and riboflavin in the tomato plant has been investigated, and it has been found that:

1. The immature leaves and stem have the highest concentration of these vitamins.

2. In mature leaves practically all of the vitamin is in the blade of the leaf.

3. The roots have as much riboflavin as the middle part of the plant but much less than the apical part of the plant.

4. The roots have considerably less thiamin than the middle part of the plant.

5. There is an increase in the thiamin and riboflavin in the tip of the tomato plant as it increases in age or size, at least up to 4 to 5 months.

6. The mature leaves contain more of these vitamins than do the ripe fruits.

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THE ABSORPTION OF PHOSPHORUS AND IRON FROM NUTRIENT SOLUTIONS[1]

C. M. F R A N C O A N D W. E. L O O M I S

Two of the more popular solutions for experimental culture of plants show a striking variation in the amounts of phosphorus used. Shive's so-called best solution, R_5–C_2 (**4**) contains 2.45 gm. per liter or 0.018 mols of KH_2PO_4, partly as a source of phosphorus, but more perhaps as an acid buffer tending to maintain the solution at pH 4.5–5.0. Hoagland's 1940 solution (**2**) on the other hand contains only 0.068 gm. per liter or 0.0005 mols of the same salt. The Shive solution thus contains 36 times as much phosphorus as the equally successful Hoagland solution. As a portion of a general study of ion balance in nutrient solutions we have compared the growth, color and phosphorus absorption of several plants in these mixtures and in two experimental solutions; the first ("X") somewhat resembling the Hoagland solution, but carrying more than half of its nitrogen as NH_4NO_3, and the second ("P") being a modification of Zinzadzé's buffered solution (**6**) carrying a moderately high concentration of phosphorus as a colloidal precipitate of the tricalcium salt and with the nitrogen again added as NH_4NO_3.

Methods

Plants were grown in quart mason jars with cork stoppers (**3**). Four seedlings in each jar were thinned to two to obtain maximum uniformity. Five replicates were used for each treatment and some of the experiments were repeated as many as five times. Corn (*Zea mays*), broccoli (*Brassica oleracea* var. *italica*), soybeans (*Glycine max*), tomatoes (*Lycopersicon esculentum*), sunflowers (*Helianthus annuus*), cotton (*Gossypium hirsutum*), and rice (*Oryza sativa*) were used in a main test of growth rates in April and May of 1945; corn, soybeans, and rice were used in June and July of the same year for studies of phosphorus absorption. Some of the plants were grown to fruiting, but, because of the small size of the culture jars, most of the work, including the phosphorus analyses, was done with plants 4 to 6 weeks old.

Concentrations of the salts used in grams per liter and of the various ions in millimols are shown in table I. Iron was furnished by 10 ml. of a 0.1% solution of ferric tartrate at each change of solutions and by 1.0 ml. additions of the same solution one to three times a week as required. All solutions received 1.0 p.p.m. of boric acid and 0.5 p.p.m. of $ZnSO_4$ at each change. After the seedling period of 2–3 weeks, solutions were changed at

TABLE I

CONCENTRATIONS OF SALTS IN GRAMS PER LITER AND OF IONS IN MILLIMOLS FOR FOUR NUTRIENT SOLUTIONS

SALT OR ION	HOAGLAND 1940 ("H")	SHIVE R_5–C_2 ("S")	EXP. SOL. "X"	EXP. SOL. "P"
	gm./l.	gm./l.	gm./l.	gm./l.
NH_4NO_3			0.25	0.286
KNO_3	0.506		0.25	
$Ca(NO_3)_2$	0.591	1.228	0.25	
KCl			0.25	0.50
$MgSO_4$	0.247	3.697	0.50	0.50
$Ca_3(PO_4)_2$				1.00
KH_2PO_4	0.068	2.450	0.10	
Total	1.412	7.375	1.60	2.286
	m. mols	m. mols	m. mols	m. mols
Ca^{++}	2.5	5.2	1.1	3.2
Mg^{++}	1.0	15.0	2.0	2.0
K^+	5.5	18.0	6.6	6.7
NH_4^+			3.1	3.6
NO_3^-	7.5	10.4	9.7	3.6
SO_4^{--}	1.0	15.0	2.0	2.0
HPO_4^-	0.5	18.0	0.7	3.2

weekly intervals, except that solutions for larger plants (6–10 weeks) were changed at intervals of 3–5 days.

GROWTH OF PLANTS

The size ranking of plants started April 4–11 was estimated on May 10 and again on May 23. Differences between the best and poorest cultures

TABLE II

RANKING FROM BEST TO POOREST OF SEVEN SPECIES IN FOUR SOLUTIONS. EXPERIMENT STARTED WEEK OF APRIL 4

PLANT	RANK ON BASIS OF TOP AND ROOT GROWTH			
	FIRST	SECOND	THIRD	FOURTH
May 10, 1945				
Corn	H	X	P	S
Broccoli	P	X	H	S
Soybeans	P	X	S	H
Tomatoes	P	X	S	H
Sunflowers	P	X	H	S
Cotton	P	S	H	X
Rice	P	X	S	H
Scores	P 26	X 19	H 12	S 12
May 23, 1945				
Corn	H	P	S	X
Broccoli	X	P	H	S
Soybeans	P	X	H	S
Tomatoes	P	X	S	H
Sunflowers	P	H	X	S
Cotton	P	H	S	X
Rice	X	P	S	H
Scores	P 25	X 18	H 16	S 11

were slight in tomato to marked in soybean and broccoli (fig. 1).   Rankings together with a score obtained by assigning a value of 4 for first rank, 3 for second, etc., are shown in table II, and six of the plants are shown in figure 1.

On the basis of the scores in table II the "P" solution containing NH_4NO_3 and $Ca_3(PO_4)_2$ was the best for the plants tested, while Shive's R_5-C_2 ("S")
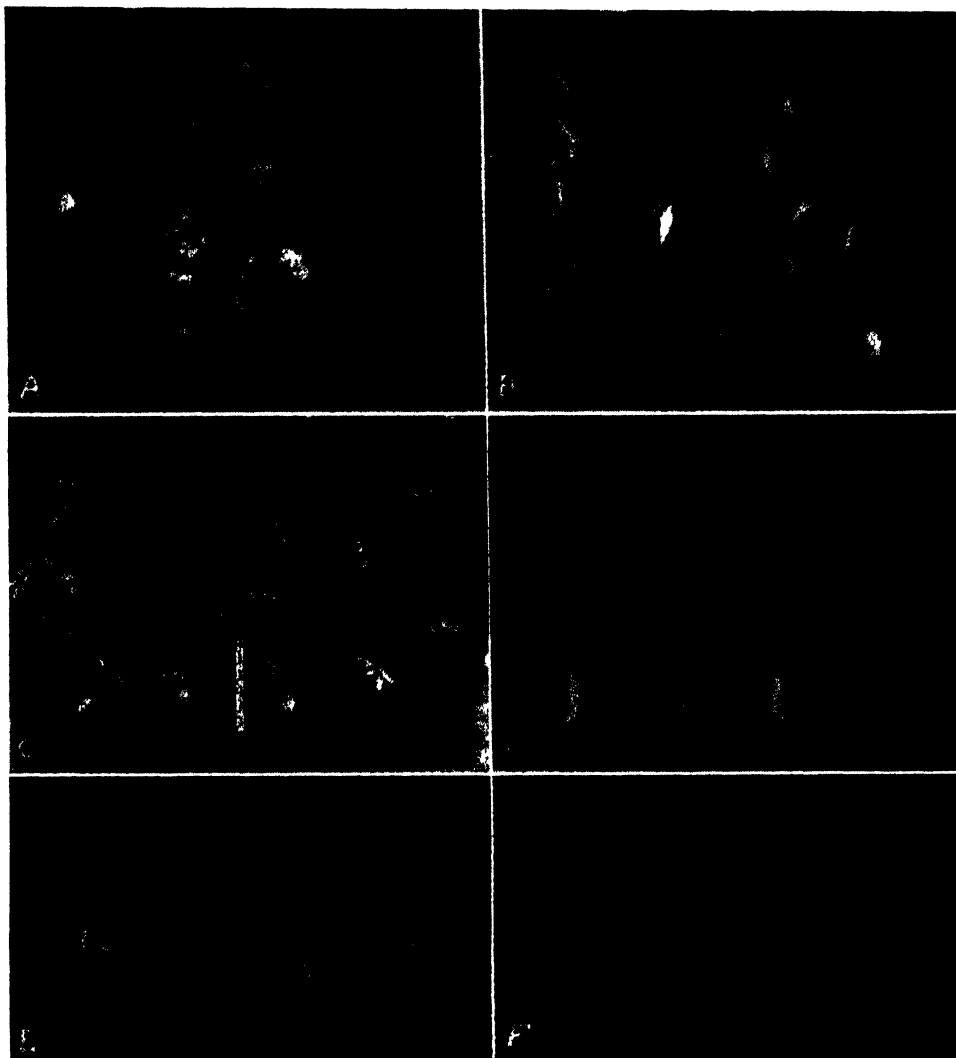
FIG. 1.   Six species grown in four nutrient solutions.   A, soybeans; B, corn; C, tomatoes; D, rice; E, broccoli; F, cotton.   Left to right in each photograph: Hoagland's solution ("H"), Shives R_5-C_2 ("S"), a buffered solution ("P") and an unbuffered solution with NH_4NO_1 ("X").   Plants started April 4–11, photographed June 15.

rated only one second place in the upper brackets.   There was much evidence, however, that the "best" solution changes with the light and temperature conditions (1), with the plant, and perhaps most rapidly with the age of the plant.   "X" was a good solution for young corn plants, but they soon developed root rots in the excessive acidity produced.   On the other hand, larger plants did reasonably well when returned to this solution (fig. 1B).

Small corn in Hoagland's ("H") solution is chlorotic and may die unless especial attention is given to the iron supply. Even badly chlorotic plants recover quickly, however, if the phosphorus is omitted from the solution for the first half of the week. Figure 2A shows this effect. The solutions were identical except for the time of adding phosphorus. Soybeans also do poorly in "H" solution, but can be carried by delaying the phosphorus (fig. 2B). This principle was used by WEISS (5), working in this laboratory. Chlorosis was induced in genetic lines of soybeans to be tested for efficiency of iron absorption by adding moderate quantities of KH_2PO_4 to a large culture tank. A differential chlorosis developed among the lines which disappeared as the phosphorus was exhausted and more iron was added to the solution.
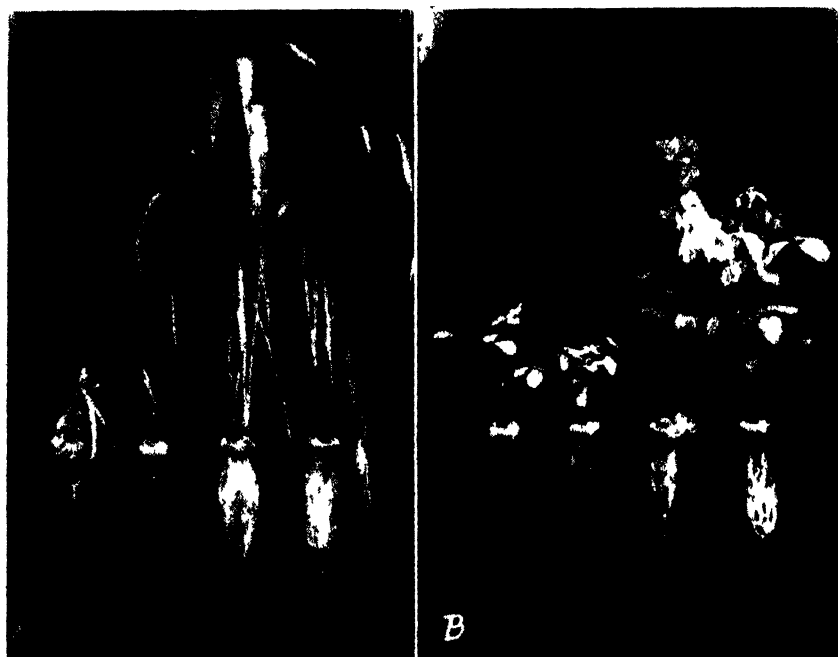
FIG. 2. Effect of alternating phosphorus and iron in Hoagland's solution. A, corn; B, soybeans; left in each photograph, phosphorus and iron added together at solution changes; right, phosphorus addition delayed 2–4 days to permit unhindered iron absorption.

The effect of phosphorus in causing chlorosis in "H" but not in "S" solution, which contains 36 times as much phosphorus, is explainable on the basis of pH. The pH's of fresh and used solutions in table III show that the large quantity of KH_2PO_4 tended to maintain an acid reaction favorable to iron absorption. Modified Shive solutions containing intermediate concentrations of acid phosphate are less successful. In Shive's original paper (4), solutions in the R_1 series contained KH_2PO_4 at 0.0036 mols; series R_2 at twice this concentration, R_3 at three times, etc., to R_7. None of the R_1 or R_2 solutions was rated good while R_4's and higher were mostly good to excellent, and three solutions, R_3–C_3, R_4–C_5, and R_7–C_2, were not significantly poorer than R_5–C_2.

TABLE III

HYDROGEN ION CONCENTRATIONS (PH) OF FRESH AND USED NUTRIENT SOLUTIONS

SOLUTION	FRESH SOLUTION	USED SOLUTION, 3–4-WEEK PLANTS	USED SOLUTION, 5–6-WEEK PLANTS
Hoagland 1940	5.20	5.80–6.35	5.40–6.55
Shive R_5–C_2	4.45	4.85–5.65	4.45–5.70
Exp. sol. ''X''	5.25	3.55–4.40	3.70–5.70
Exp. sol. ''P''	6.10	5.50–6.30	5.15–5.60

Ammonium nitrate was added to solutions ''X'' and ''P'' as a neutral source of nitrogen. We were very considerably surprised, therefore, to find that the pH of the unbuffered solution ''X'' dropped as low as pH 2.9 and always went below 4.0. The cause of the acidity was shown to be the preferential absorption of ammonia ions by all of the plants studied at all stages of growth up to early fruiting. Ammonia was absorbed faster than NO_3^- from the unbuffered ''X'' solution, the buffered ''P'' solution or pure NH_4NO_3. Corn and cotton roots (fig. 1F) were injured in these acid solutions but soybeans made excellent growth with a very dark green color (fig. 1A). Young plants sometimes became chlorotic in ''X'' because they did not absorb nitrogen fast enough to develop an acid reaction. With large plants nitrogen absorption was so rapid that the acid phase was ended on the second or third
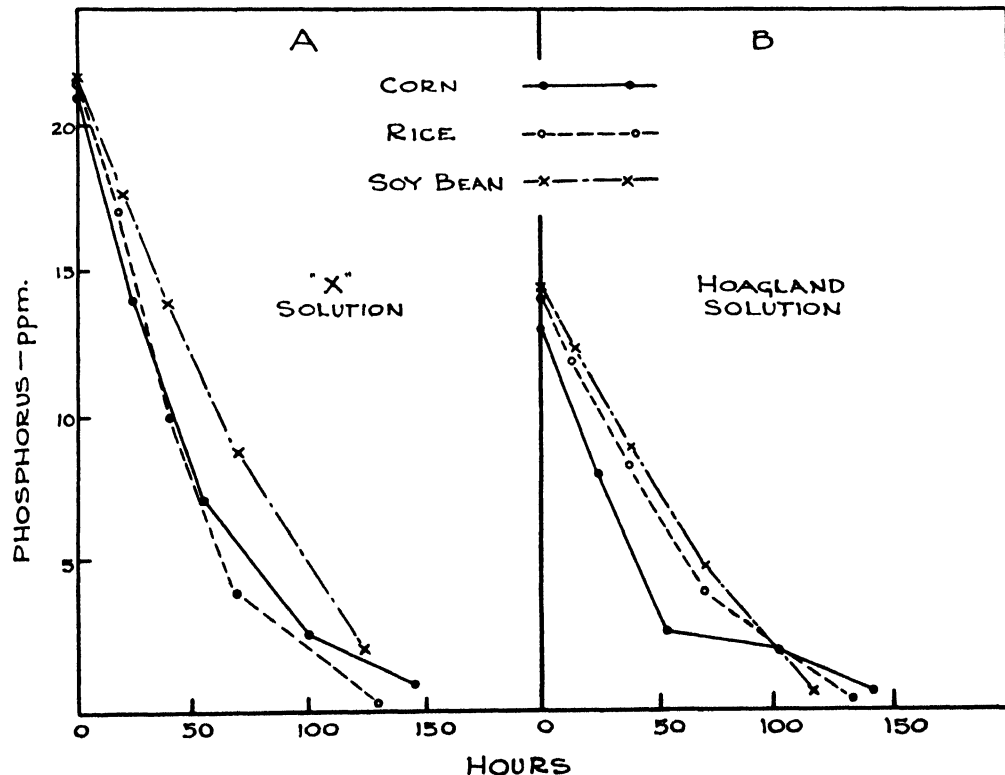
FIG. 3. Phosphorus absorption by three species grown in ''X'' and Hoagland solutions.

day by nearly complete absorption of both NH_4^+ and NO_3^-. In the "P" solution the $Ca_3(PO_4)_2$ prevented low pH values. At the lower pH's shown for this solution in table III, phosphorus became more soluble (fig. 5) and some tendency toward chlorosis was observed.

THE ABSORPTION OF PHOSPHORUS

The apparent interaction of phosphorus and pH in iron chlorosis led to a detailed study of PO_4 absorption from the four solutions. The results are shown graphically in figs. 3, 4, 5. The "H" and "X" solutions (figs. 3A and B) starting respectively with 14 and 21 p.p.m. of phosphorus dropped to 1 p.p.m. or less in 5 or 6 days. Essentially all of the 0.068 or 0.100 gm.
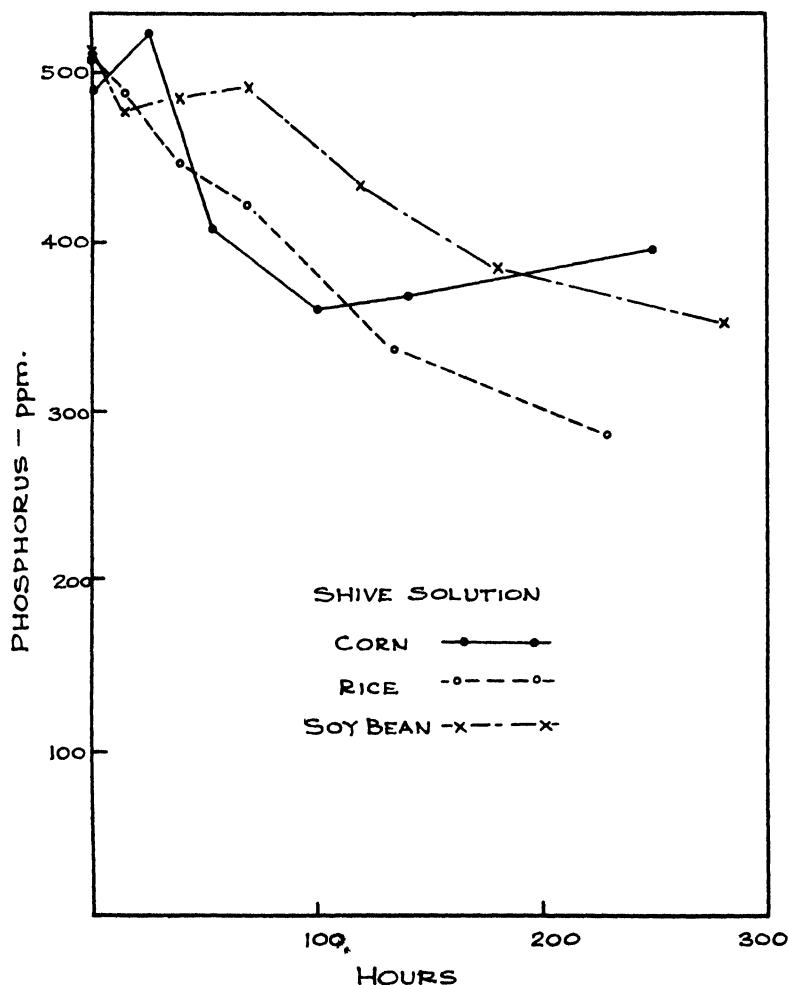
FIG. 4. Phosphorus absorption by three species grown in Shive solutio...

of KH_2PO_4 added was absorbed. The 500 p.p.m. of phosphorus in the "S" solution was reduced to about 300 p.p.m. in a week (fig. 4) with a phosphorus absorption 10 or 15 times that in the "X" and "H" solutions. The fact that such heavy absorption did not seriously interfere with iron utilization is evidence that the interference observed with the "H" solution occurred out-

side the plant.   Soluble phosphorus concentrations in the "P" solution, which contained a large excess of colloidal $Ca_3(PO_4)_2$, started at about 10 p.p.m. and climbed to 20–30 p.p.m. on the second and third days (fig. 5) as some of the insoluble phosphate was brought into solution by accumulating HNO_3 from the differential utilization of NH_4NO_3.   Soluble phosphorus then tended downward but remained above 10–20 p.p.m. after 10 days.   The "P" solution has been best for small plants with slow NH_4^+ absorption so that the pH is held near 6.0.   Very little iron chlorosis develops in these cultures in
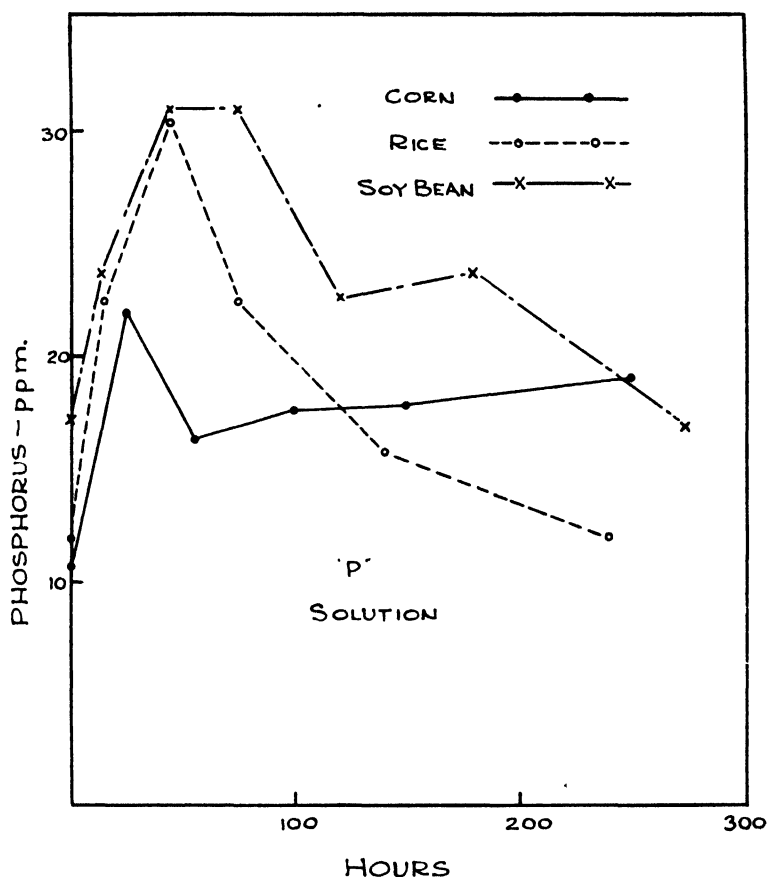
FIG. 5.   Phosphorus absorption by three species grown in the $Ca_3(PO_4)_2$ buffered "P" solution.

spite of the high pH and phosphorus, suggesting that it is the dibasic or more probably the monobasic phosphate ion which precipitates the iron.   The "P" solution would probably be improved for older plants by using some $Ca(NO_3)_2$ along with $Ca(Cl)_2$ in forming the $Ca_3(PO_4)_2$.   The result would be a partial replacement of KCl by KNO_3, a higher total nitrogen and perhaps less tendency for excess solubility of the phosphorus.

Discussion and summary

Moderate amounts of KH_2PO_4 (0.5 to 0.7 millimols) caused iron chlorosis of seedlings in solutions less acid than pH 5.5–6.0.   The trouble could be

avoided by omitting the phosphorus entirely and adding it separately after 2–4 days. Even with this modification the Knop type of solution (Hoagland, **2**) caused persistent chlorosis in soybeans and broccoli.

In the commonly used Shive solution (R_5–C_2) a large excess of KH_2PO_4 holds the pH of the solution between 4.5 and 5.0 and chlorosis is moderate to slight. The Shive solution contains 36 times as much phosphorus as the Hoagland and 6-week-old plants growing in it absorbed 15 times as much to make a slightly poorer growth. The addition of as little as 0.125 gm. per liter of NH_4NO_3 resulted in decidedly acid solutions in every experiment with nine plant species representing seven families. Initial pH's of 5.25 in an unbuffered solution fell to minimums of 3.9 to 2.9 and then rose, to pH 5.0 or 6.0 with large plants. The low pH's were shown to be due to a preferential absorption of NH_4^+ ion and the later rise to the slower absorption of the NO_3^- ion. The use of NH_4NO_3 prevented iron chlorosis with some plants, notably soybeans, but resulted in acidities which were injurious to young corn and to cotton. In work done in this series but not described above, the ''X'' solution containing NH_4NO_3 gave exceptional growth of young coffee (*Coffea arabica*), but was toxic to older plants.

It is probable that iron chlorosis has more effect on solution culture results than any other single factor, and frequently than all other factors. Iron absorption from cultures is reduced by phosphorus, probably by $H_2PO_4^-$ ions especially, at pH's of about 6.0 or higher. The use of $Ca_3(PO_4)_2$ reduces the trouble as does the use of enough KH_2PO_4 to maintain a pH below 5.5. In tank culture H_2SO_4 may be used more cheaply to accomplish the same result. In miscellaneous work with the Knop type of solution chlorosis can be reduced or prevented by omitting phosphorus from the solution and adding it separately 2–4 days later after iron has been absorbed.

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SELECTIVITY OF 2,4-D AND SINOX WHEN APPLIED TO SOIL

L. L. DANIELSON

(WITH ONE FIGURE)

The use of 2,4-D (2,4-dichlorophenoxyacetic acid) and Sinox (sodium dinitro ortho cresylate) for the control of weeds in broadleaf crops has not proved feasible in any general way. Evidence is accumulating, however, to show that certain crops may be treated with these materials in some stages of development, and in some cases during dormancy, without producing apparent injury. Crop plants of this type, however, are very limited in number.

The application of these chemicals to the soil to control weeds in field seed beds seemed to present a possibility for extending their use. Accordingly, a preliminary experiment was conducted in 1946 to canvass the possibilities in such a method of application.

The trials were conducted on a Sassafras loam prepared as for seeding corn or lima beans. Sinox in concentrations of one to four gallons per acre and 2,4-D (70% sodium salt) in concentrations of 1 to 4 pounds per acre were applied as a spray on this freshly prepared soil. The chemicals named were applied on plots 8 by 50 feet square in sufficient water to provide 500 gallons per acre to make possible the uniform distribution of the chemicals.

TABLE I

EFFECT OF SOIL TREATMENTS ON WEED POPULATIONS EXPRESSED AS NUMBER OF BROADLEAF AND GRASS PLANTS PER SQUARE YARD*

SINOX—GALS. PER ACRE	1	2	3	4
Broadleaf plants				
Av. 3 areas	47.3	8.3	2.6	0.0
% control	46.1	90.6	96.8	100.0
Grass plants				
Av. 3 areas	169.6	148.3	98.6	75.6
% control	10.4	21.7	11.6	31.5
2,4-D—LBS. PER ACRE	1	2	3	4
Broadleaf plants				
Av. 3 areas	26.6	27.6	28.3	40.3
% control	67.0	65.8	81.1	73.1
Grass plants				
Av. 3 areas	16.6	21.0	16.3	9.6
% control	85.0	81.0	88.3	93.1

* Principal broadleaf weed was carpetweed (*Mollugo verticillata*). Principal grass was crabgrass (*Digitaria sanguinalis*). 2,4-D used was the sodium salt in 70% concentration. Soil treated 7/1/46. Populations counts made 8/22/47.

The applications were made in the first week of July 1946 and were evaluated 7 weeks later. The treated areas were not cultivated during this period.

Periodic examination of these plots revealed a striking difference between the plant selectivity of the two chemicals as applied to the soil in the manner described. As is generally known, the selectivities of these materials are very similar when applied to the foliage of growing plants. Evaluation of these plots 7 weeks after treatment, table I, showed that the 2,4-D was toxic to both the grasses and the broadleaf weeds, whereas the Sinox was only slightly toxic to the grasses but very toxic to the broadleaf weeds. Figure 1 shows the effective control of grasses and broadleaves of a 4-pound-per-acre application of the 2,4-D.

FIG. 1. Check plot (1) at left above. Plot (2) at right which received 4 pounds per acre of the sodium salt of 2,4-D (70%) applied to the soil in 500 gallons of water per acre.

The toxic effects of these materials were rather quickly dissipated in the soil as indicated by the normal growth in all treatments of a planting of Henderson bush lima beans and Golden Cross sweet corn made ten days after the chemicals were applied to the soil. The effective rainfall during the interval between treatment and planting was 3.75 inches.

The results of these preliminary trials are considered as indicative rather than conclusive because of their limited nature in terms of weather and soil variations.

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SOLUBLE SOLIDS CONTENT OF DIFFERENT REGIONS
OF WATERMELONS

JOHN H. MACGILLIVRAY

Several workers have used a hand refractometer to determine the soluble solids content of watermelons, *Citrullus vulgaris* Schrad. These values appear to be closely related to the sugar content of the melon. Judging from the data of PORTER, BISSON, and ALLINGER (2) in Klondike watermelons, 85% of the soluble solids consists of total sugars. Thus, the hand refractometer reading has been used as a measure of quality. An accurate method of determining soluble solids is especially valuable in studying the effect of this quality factor, both in improvement programs and in work on the effect of environment on quality. An accurate method of sampling the melon is also of critical interest. In 1934 TUCKER (4) noted the variability of different sections of a watermelon in soluble solids content. Since Tucker's data were limited to observations on one melon, it has seemed desirable to repeat the work, using a larger number of fruits.

In 1938, watermelons from an irrigation experiment were used in this project. Both nonirrigated and heavily irrigated melons of the Klondike variety were included. Previous studies (1) have shown that, at Davis, California, irrigation does not greatly affect soluble solids content. All comparisons in this paper were between readings made on the same melon. For the determinations, the melon was placed with the ground spot on a table and cut into equal halves vertically from stem to blossom end. The areas shown in figure 1–B were then sampled. Sample areas in the red flesh were given numbers, whereas those close to seed regions were indicated by small letters of the alphabet. Next, the sampled half was laid on the table with the cut surface up. The melon was cut vertically from stem to blossom end into two quarters. The cut surface of the quarter having the ground spot was then sampled (fig. 1–A). All samples were obtained from the edible flesh even though close to seed or rind. Juice was expressed by hand from a cube of the flesh about one-half inch on each side. Soluble solids content of each sample was determined with a hand refractometer. A scale on this instrument gives the solids percentage of pure sucrose solutions. Readings thus obtained are called, in this paper, "soluble solids." Values found are the averages obtained from twenty-three melons used in this experiment (fig. 1 and table I).

Edible flesh was removed from the two quarters used for the small samples, and the juice was extracted by hand, pressing the flesh in a piece of cheesecloth. The soluble solids content of this juice was considered representative of the whole melon.

In each of the watermelons twenty-three areas were sampled. Table I shows these values, arranged in a descending order from the largest to the smallest. The data indicate a marked decrease in the sugar content as the samples approach the rind from the seed area. High soluble solids are found in the center flesh of the melon, including the surrounding tissue near the seed area. These values which vary from 12.85% to 12.25%, average 12 59%
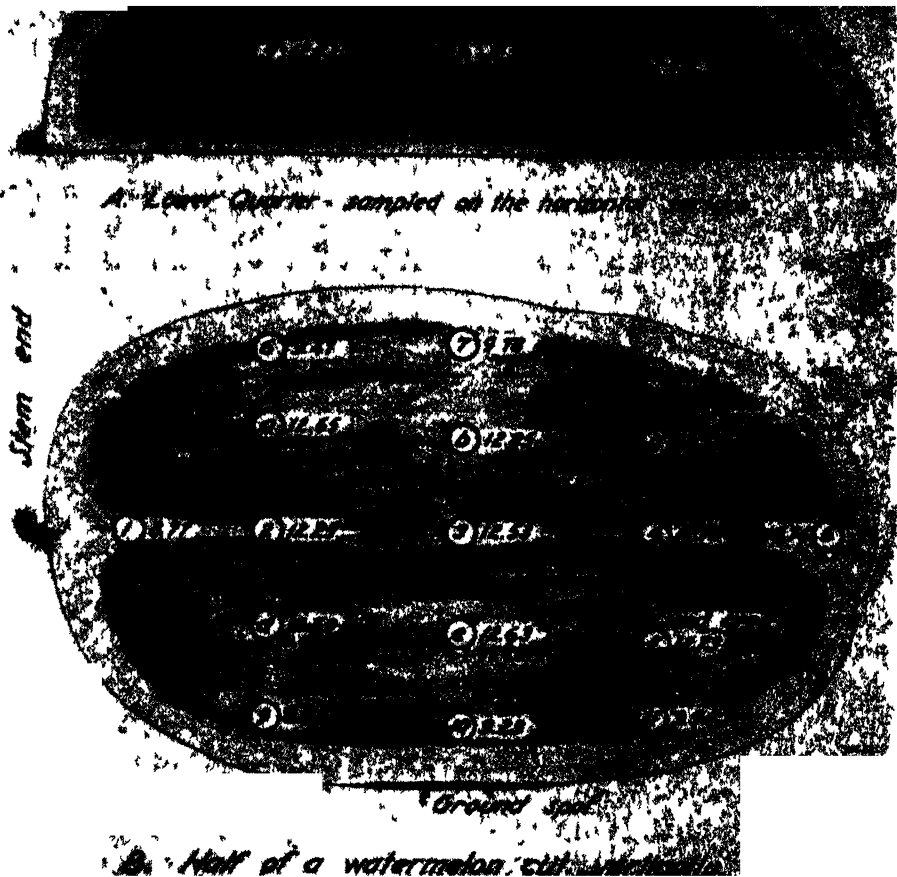
FIG. 1. The percentage of soluble solids in the different areas of a watermelon.

soluble solids. Flesh near the rind is low in soluble solids, varying from 10.74% to 8.57% and averaging 9 33%. The blossom end is almost 2% higher in solids than the stem end; likewise the samples near the upper rind· are higher than those near the ground. The watermelon fruit seems to be consistently variable in soluble solids content.

To compare or improve varieties as to quality, one must have some method of obtaining an adequate sample. For some light on this problem, one may

compare the results obtained from the individual samples and the soluble solids content of the juice from the half melons. Juice from the halves averaged 11.23% soluble solids. The average for all the small samples (1–14, a–i) is 11.06%. When the average value of the melon was determined by averaging the twenty-three (fig. 1–A and 1–B) small samples and compared with the value for the half melons, there was found (by Student's method) a significant difference. Since the odds in this case were 103 to 1, evidently the twenty-three samples include too large a proportion of those near the rind. On the other hand, averaging the seventeen samples from the half (1–11, a–f) of the melon, one finds a value of 11.22%, which is not significantly different from our value for the half melon sections (11.23%). If

TABLE I

SOLUBLE SOLIDS CONTENT OF DIFFERENT SECTIONS OF A WATERMELON FRUIT

| | MELON SECTION—SEED OR CENTER | | MELON SECTION—NEAR RIND | |
MELON	SECTION DESIGNATION, FIGURE 1	SOLUBLE SOLIDS*	SECTION DESIGNATION, FIGURE 1	SOLUBLE SOLIDS*
		%		%
Seed	B–b	12.85	B–5	10.74
Center	B–4	12.78	B–8	10.21
Seed	B–f	12.73	B–11	9.94
Seed	B–a	12.65	B–7	9.78
Seed	B–e	12.63	B–6	9.47
Seed	B–d	12.59	B–10	9.23
Seed	A–h	12.55	B–1	8.77
Center	B–3	12.53	B–9	8.72
Seed	B–c	12.53	A–14	8.63
Seed	A–i	12.47	A–13	8.59
Seed	A–g	12.43	A–12	8.57
Center	B–2	12.25		

* Arranged in descending order.

individual regions are compared within a given melon, a difference of about 0.6% soluble solids is necessary for a significant difference, with odds of 19 to 1.

None of the twenty-three areas selected had an average value of 11.23%. Twelve of the percentages are higher and eleven are lower. If, for sampling, one must choose a sector that is similar in composition to all the juice, it will have to be located between the rind and the seed region. Such a sector might lie between samples 4 and 5. Area 3, which has been used for sampling many melons, is desirable from the standpoint of the small variation from the surrounding tissue. One should remember that this area is 1.3% higher in soluble solids than the average.

In a fruit so variable as the watermelon, the choice of one area for sampling must involve certain dangers of inaccuracy. The data obtained in this experiment resemble those published by TUCKER (4) on the variability of the readings. According to Tucker, the center flesh varied from 8.1% to 14.9%

soluble solids, and the flesh near the rind from 4.1% to 6.8% soluble solids. SCOTT and MACGILLIVRAY (3) have shown a similar amount of variability in cantaloupe fruits, *Cucumis melo* L.

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NOTES

Chicago Meeting.—Plans for the annual meeting of the Society include the usual dinner to be held at the Congress Hotel which is near Headquarters at the Stevens; the price will be $3.50 per plate. In the light of present arrangements for session rooms it is imperative that all members of the Society register since the more Plant Physiologists who register, the larger the proportion of room rentals for our Society that will be paid by the A.A.A.S. Full publicity has been released relative to registration and the General Program may be obtained from the offices of the A.A.A.S. at 1515 Massachusetts Avenue, N.W., Washington 5, upon payment of the usual fee of two dollars. This program is planned for release on December 1; early registration is suggested.

Purdue Section Meeting.—On July 24 and 25, the Purdue Section of the AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS celebrated the Twenty-first Anniversary of its organization in 1926. DR. E. J. LUND, University of Texas, gave three lectures on the general subject of "Bioelectrical Phenomena and Cell Correlations" followed by a round table discussion of the results that he presented. The meetings were opened with registration during the morning of July 24 and inspection of a number of interesting projects in the biological laboratories. Forty-five people attended the noon luncheon and heard a very interesting and enlightening discussion concerning the proposed Institute of Biological Sciences by DR. J. FISHER STANFIELD. About 90 attended DR. LUND's first lecture on "Pattern and Variations of the Bioelectric Field" and the same number heard his second lecture at the dinner meeting on Thursday evening on "Orientation of Growth and Polarity of the Field." About 70 were present to listen to a discussion Friday morning on "Electrochemical Basis of the Electric Polarity of the Cell" and participated in a round table discussion following the lecture. The meetings were climaxed with a trip through Turkey Run State Park which fifty attended on Friday afternoon. Officers for next year are DR. R. E. GIRTON, Chairman and DR. H. H. KRAMER, Secretary-Treasurer.

General Botany Laboratory Book.—EDWARD M. PALMQUIST and LOREN C. PETRY. W. B. Saunders Co., Philadelphia. 174 pages. $2.25. This latest addition to the list of manuals for introductory plant science represents thirty-five exercises which are adaptable to varied types of courses. Many questions, with space for answers, provide motivation for closer study of materials; prepared drawings for labelling are ample. With a coiled wire binding it is intended that the book should be turned in for examination as a unit rather than as separate exercises; certain record sheets, however, are to be torn out and handed in. Winter keys to common trees are included in the appendix. This manual is clearly written and illustrated, represents

time-proven experiments, and should be well received as a useful guide in the laboratory.

Abdel Galil El Gawadi.—DR. ABDEL GALIL EL GAWADI was born May 4, 1908 at Faraskur, Egypt. Educated in Egypt, he entered Fouad I University of Cairo in 1926 and continued his education at Cambridge University, England where he received the Doctor of Philosophy degree in 1935. Here he met his future wife, Hilda Robisher, and two daughters, Aida and Nadya, were born of his marriage in 1936.

DR. GAWADI returned to Egypt to continue his career at Fouad I University as a lecturer in Plant Physiology where he remained until 1942 when he was transferred to the new Farouk I University of Alexandria. Coming to the United States in 1946 he affiliated with the Brooklyn Botanic Garden as a Research Fellow with the general problem of Leaf Abscission. A promising career was brought to an abrupt end by his death on August 21, 1947.

While DR. GAWADI had enjoyed only limited opportunities to enlarge his circle of friends in this country he was highly esteemed by his colleagues. Clarity of vision, a cordial·manner, and scrupulous honesty were attributes enjoyed and respected by all who knew him.

Radioactive Tracers in Biology: An Introduction to Tracer Methodology.—MARTIN D. KAMEN. Academic Press Inc., New York, 1947. 231 pages. $5.80. This manual reviews concepts in nuclear physics which are basic to proper application of tracer methods in biology and provides a critical survey of existing procedures with potentialities and limitations thereof for general biological research. The author presents the actual data of his own and many other original investigations employing radioactive isotopes. Reference is also made to the use of stable isotopes in tracer studies though somewhat briefly in the interest of clarity. A survey of special topics, concerning medical applications of radioactive isotopes is included in the final section of the book.

Though this book is not a compendium for experts, it provides a thorough introduction to tracer research in biology and will be of inestimable value to biochemists, physiologists, and physicians seeking orientation tracer methodology and peaceful applications of nuclear energy. The text includes discussion of atomic nuclei, radioactive isotopes and methods of their production, radiation characteristics of tracer atoms and procedures for assay thereof, followed by comments on radioactive forms of hydrogen, carbon, phosphorus, sulphur, the light and heavy metals. The author's style is lucid, the contents are very coherently organized and the text is generously illustrated. There are numerous yet carefully selected citations in extension as well as in support of the text, enabling those who desire more extended knowledge of current aspects of radiobiology to obtain ready access to the most authoritative and recent publications in the field. Author and subject

indices make the contents available for rapid reference. The book serves a most useful purpose for biologists in collation and evaluation of a widely scattered literature as this relates to theory, preparation, testing and results in tracer research. The author and publisher are to be commended on the timeliness and general excellence of the book.

Fatty Acids, Their Chemistry and Physical Properties.—K. S. MARK-LEY. Interscience Publishers, 215 Fourth Avenue, New York 3. 668 pages. $10.00. This monograph deals with the chemistry and technology of fats, oils, and related substances. The subject matter is well organized and brings together in an accessible form many data pertaining to the chemical reactions and physical properties of the fatty acids, with particular stress on the long chain fatty acids. With preliminary chapters on the history, classification, nomenclature, and isomerism of fats, waxes, and fatty acids, subsequent chapters discuss physical properties, chemical reactions, synthesis, isolation, and identification of fatty acids. Many new aspects of spectral properties, pyrolysis, autoxidation, and the biosynthesis of fatty acids are presented.

Although the book is principally intended for chemists much vital information will be of interest to the plant physiologist, particularly the section on synthesis in plants. The excellent organization of the material will obviate much laborious search of the literature for those interested in this type of research.

Bioelectric Fields and Growth.—E. J. LUND and COLLABORATORS. University of Texas Press, Austin, Texas. 391 pages. $7.50. This book collates the major research of the laboratories of physiology and biophysics at the University of Texas. It is a very helpful interpretation of experimentation by the outstanding leaders of the rapidly expanding field of electrophysiology.

Theoretical aspects of the subject matter are closely integrated with the experimental data, presented under seven separate headings. The first considers the topic of Electric Fields of the Cell and Polar Cell Aggregates, the second and third deal with the Effects of Gravity on the Electric Correlation Field in the Oat Coleoptile and the Spontaneous Variations in the Electric Field Potentials of the Root of the Onion. The subsequent sections treat the topics of Polar Bioelectric Fields and Polar Growth Under Conditions of Applied Electric Fields, Biocoulometry, General Discussion of the Results and Significance of the Results by the senior author. The final section consists of a concise bibliography of Continuous Bioelectric Currents and Bioelectric Fields in Animals and Plants with index by H. F. Rosene.

Acta Scandinavica.—Volume I, 1947. Published by Chemical Societies in Denmark, Finland, Norway, and Sweden. Distributed by Einor Munksgaard, Copenhagen, Denmark. Annual Subscription, $8.50 plus postage.

The Journal Acta Chemica Scandinavica is again readily available to American subscribers. Dealing with the work of Scandinavian chemists in the fields of general, physical, inorganic, organic, and biochemistry, this publication appears ten times yearly. All articles and contributions are printed in one of three languages: English, French, or German. Aquaintanceship with this journal should be especially helpful to the American plant physiologist in keeping abreast with recent research of the Scandinavian countries.

Advances in Enzymology and Related Subjects of Biochemistry.— Volume 7, 1947. Edited by F. F. NORD, Interscience Publishers, 215 Fourth Avenue, New York 3. 665 pages. $8.75. Of special interest to physiologists in the newest volume of this distinguished series will be the sections on: Permeability and Enzyme Reactions by S. C. BROOKS; Properties of Protoplasm with Special Reference to the Influence of Enzyme Reactions by WILLIAM SEIFRIZ; Applications of Radioactive Indicaters in Turnover Studies by G. HEVSEY of Stockholm; Bacterial Luminescence by FRANK H. JOHNSON; Heme-Linked Groups and Mode of Action of Some Hemoproteins by HUGO THEORELL of Stockholm; Interrelations in Microorganisms between Growth and the Metabolism of Vitamin-like Substances from Fungi and Green Plants by FREDERICK KAVANAGH; and Recent Progress in Industrial Fermentations by F. M. HILDEBRANDT. This volume also contains a cumulative index to Volumes I to VII as well as author and subject indices to Volume VII.

Annual Review of Biochemistry.—Volume XVI, 1947. Annual Reviews, Inc. Stanford University Postoffice, California. 740 pages. $6.00. Of the twenty-five sections of the most recent volume of this review journal, the following will be of particular interest to physiologists: Biological Oxidations and Reductions; Proteolytic Enzymes; Metabolism of Lipids, Carbohydrates, Proteins and Amino Acids; Antioxidants; Nitrogenous Constituents of Plants; Mineral Nutrition of Plants; Growth Substances in Higher Plants and Bacterial Metabolism. The volume contains complete, cross-referenced author and subject indices.

Plant and Soil.—Volume I, 1947. E. G. MULDER, Editor. A new periodical published by Martinus Nijhoff Company, The Hague, Holland. 400 pages. Subscription $7.50 per volume. A new important international periodical, "Plant and Soil," will be issued towards the end of 1947. It is devoted to the study of plant nutrition, plant chemistry, and the related subjects of soil science, soil microbiology, and soil-borne plant diseases. Realizing that the increasing demand for intensive crop production all over the world necessitates a quick exchange of the results of scientific research between the workers in this field, a number of outstanding scientists from different countries have decided to collaborate in establishing this new journal. Among the Editors we read the names of GÄUMANN of Zürich; LUNDE-

GÅRDH, PENNINGBY, and MELIN of Uppsala; HOAGLAND of Berkeley; KON-INGSBERGER of Utrecht; WAKSMAN of New Brunswick; WESTERDIJK of Baarn; VERTANEN of Helsinki, and many other outstanding scientists. Original contributions in English, French or German may be sent to the secretary of the Board of Editors, DR. E. G. MULDER, Agricultural Experiment Station, Eemskanaal ZZ. 1, Groningen, Holland.

Statistical Analysis of Biology.—K. MATHER. Interscience Publishers, 215 Fourth Avenue, New York 3. 267 pages. $5.00. This book is a valuable analytical tool to plant scientists and research workers in general. The author outlines the scope of the various statistical methods and their interrelationship. Representative analyses and examples in the text show how statistical evaluations are applicable to the data of the biological sciences. The subject matter dealing with such basic principles of statistics as Probability and Significance, Distributions, Tests of Significance, The Interrelationship of Two Variables, Polynomial and Multiple Regressions, and Correlations is couched in a language readily understood by the non-mathematician. A glossary of terms also facilitates acquaintanceship with the vocabulary and mathematical symbols used in statistics. The volume consists of 13 chapters with an individual list of references for each subject. A set of conversion tables and a general index are also included.

Methods of Vitamin Assay.—Prepared and Edited by the Association of Vitamin Chemists, Inc. 1947. Interscience Publishers, 215 Fourth Avenue, New York 3. 189 pages. $3.50. This monograph meets the need for a concise yet critical compilation of standardized methods of vitamin analysis. Methods are presented for analysis of foods, feedstuffs, pharmaceutical, and biological products. The most accurate and widespread methods for determination of the major vitamins are listed along with selected references to other methods. Separate bibliographies pertinent to the particular subject are included with each individual chapter.

Technological and Physical Investigations on Natural and Synthetic Rubbers.—A. G. WILDSCHUT. 1946. Elseveir Publishing Company, Inc., New York. 173 pages. $3.00. This book is one of a series of monographs on the progress of research in Holland during the war. The subject matter comprises technological investigations and physical investigations on natural and artificial rubbers and related components as carried on in the Netherlands from 1939 to 1944. Though the book is intended primarily as a reference for rubber technologists, this volume will be of interest and use to plant scientists as a compilation of much important research on rubber carried on during World War II.

[1] Papers published in PLANT PHYSIOLOGY are indexed in The Agricultural Index,
The W. H. Wilson Co., New York.